

STUDIES OF HORMONES IN RELATION TO
GENETIC MERIT OF DAIRY CATTLE

by

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ABSTRACT.

Selection of cattle for increased milk yield is largely done by progeny testing of dairy bulls. This is both costly and protracted. Research into the physiology of lactation is important (i) because it may be possible to identify a heritable physiological component of production which might be used to aid selection, thus reducing costs per unit genetic gain (ii) because a deeper understanding is required of the relationships between the physiological processes underlying milk production and those affecting milk quality, beef characteristics, reproduction or disease resistance. Literature on the endocrinology of lactation is reviewed, with emphasis on the control of energy metabolism and the partitioning of energy between milk and body tissue. Insulin, thyroxine (T_4) and triiodothyronine (T_3) are known to be important in these processes. Three experiments are described in which plasma levels of these hormones were measured in cattle of known differing genetic merit for milk production.

In experiment 1, blood samples were collected from 180 British Friesian bulls awaiting the results of their progeny tests at 3 Milk Marketing Board stations. Levels of each hormone, corrected for station and age of bull, were regressed on breeding value for the 120 bulls whose test results were known after $2\frac{1}{2}$ years. None of the regressions was significantly different from zero.

In experiment 2, blood samples were taken at 12 week intervals from Friesian and Jersey calves, of both sexes, which were the progeny of sires with

either very high or very low breeding values for milk yield. Hormone levels were analysed by the method of least squares. There were no significant effects of breed, sex or high-low grouping on any of the three hormones. Age affected T3 and insulin and year-season affected insulin and T4 significantly.

In experiment 3, blood samples were taken via a jugular cannula from 6 dairy and 6 crossbred bull calves. The experiment was in two parts, the first at age 6 weeks (before rumination was established) and the second at age 16 weeks after rumination was established. Growth hormone was analysed in addition to the 3 hormones already mentioned. By analysis of serial samples estimates were obtained for each hormone of the extent of diurnal variation, repeatability, the effects of fasting and refeeding, and the effect of injecting a nutrient metabolite. Breed differences were examined. In addition, a mean level for each hormone was derived for each calf at both ages. The relationships of these means with each other and with growth rate and body weight were studied.

The main difference observed between the two breeds was in the response of insulin to feeding at age 6 weeks (preweaning) and in responses of insulin to injection of sodium propionate both preweaning and postweaning, with the crossbred calves showing significantly greater rises in insulin in all cases. Also of note were the high correlations among the thyroid hormones, body weight and weight gain at 16 weeks of age.

In the concluding section the results are discussed and prospects for future research are outlined. It is argued that the most fruitful approach for developing a physiological selection criterion for milk production may lie

in detailed measurement of patterns of physiological changes in response to imposed treatments rather than in extensive survey-type studies of basal hormone levels. It is thought that further advances may be made when it becomes feasible to make quantitative measurements of the rate of protein synthesis and breakdown in cattle.

**I declare that the work contained in this thesis is original
and is my own.**

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CHAPTER I : INTRODUCTION

Selection of domestic cattle for increased milk yield has been and continues to be extensively practised. Lerner and Donald (1966) suggested that "the positive correlation between efficiency of food use and output is one of the chief arguments for intensifying livestock production ... the intensification of farming policies is the only open-ended policy, that is, a policy of unlimited future". A major contribution towards increasing lactation yield per cow can be made by effective use of genetic variation. The present study is intended to assess whether and how the application of physiological techniques may aid this improvement process. The first three sections will describe a) the progeny testing system currently used for selection of dairy cattle in the U.K. b) possible ways in which physiological techniques may be used to make the selection more cost-effective c) evidence for some effects associated with milk yield, suggesting that further physiological research may help to clarify the links between milk yield and other economic traits. In the next sections, the physiology of milk production is reviewed. The aims of study, designed to incorporate both the objectives of the early sections and the conclusions of the review sections, are then stated.

I.1. Selection for increased milk yield.

I.1.1. Progeny testing.

Milk production is a sex-limited trait and has only a moderate heritability ($h^2 = 0.27$; Maijala and Hanna, 1974). Furthermore, selection among females is limited by their low prolificacy so that direct selection is both weak and inaccurate and can generate only small genetic responses (Rendel and Robertson,

Land (1974) developed this approach in the selection of sheep for prolificacy, a trait which again has the restraints of sex-limitation and a long generation interval. After some studies of the underlying physiology, he concluded that it may be possible to select ram lambs on the basis of their physiological characteristics in order to improve their daughters' prolificacy.

The development of a similar facility in cattle with respect to milk production could be used to advantage in the preselection of young bulls for progeny testing; either it would effectively increase the number of young bulls tested and so raise the selection differential, or it would reduce the economic cost while maintaining the selection differential. Such a preselection criterion would be the more valuable (i) the higher its heritability, (ii) the higher its genetic correlation with breeding value (iii) the easier its measurement, (iv) if measurable in young animals.

"On the borderline between operational and basic research lies the search for highly heritable markers which might be linked with or might depend on genes controlling the phenotypic expression of economic traits" (Lerner and Donald, 1966). Previous workers have sought genetic correlations between milk yield and blood groups or other polymorphic traits. The present study seeks to identify meaningful components of the physiological processes underlying milk production, rather than a chance genetic linkage between milk production and a character not otherwise relevant to it. Soller (1978) has recently concluded that "The identification of high heritability components of production could make only a minor addition to genetic improvement. Any contribution of 'marker-linked' procedures would be negligible". However, he does not specifically consider the possibility of identifying such a component in males.

1950). Since the introduction of artificial insemination (AI), it has become practicable to obtain an accurate estimate of the breeding value of males for milk production by analysing their daughters' records. In this way indirect selection of males can be used to supplement female selection. In many developed countries this has tended to become the most important path of selection; and any genetic gains accumulated among selected males can be spread rapidly and widely through the population using AI whereas an exceptional female can only make a similar contribution through her sons.

This progeny testing of dairy bulls is both costly and protracted; in England in 1976-77 the approximate expenditure for the testing programme was £40,000 per bull selected (MMB, 1977), while such a bull is likely to be 7-8 years old on completing the test. Although the selection differential could be increased and the rate of genetic response favourably affected by testing larger numbers of young bulls, the corresponding increase in testing costs would be prohibitive (Hinks, 1970).

I.1.2. Physiological aids to selection for milk yield

Lactation is sex-limited but not sex-linked; although phenotypic variation is exhibited by only one of the sexes, genotypic variation exists in both. It is possible that this genotypic variation gives rise to physiological variation even in animals where it is not being expressed as milk production; that is, genetically different animals, even calves and males, might show physiological variation similar to that which is thought to underlie phenotypic milk yield differences in mature females. Better understanding of such variation might enable selection of superior male genotypes using a physiological criterion, thus overcoming the problem of sex-limitation.

I.1.3. Possible effects associated with selection for milk yield.

Milk quality.

Selection for milk yield alone has been advocated because of the high genetic correlations reported between milk yield and efficiency (e.g. 0.81 - 0.88; Syrstad, 1966). However, selection for yield alone could give rise to other correlated responses. The genetic correlation between milk yield and fat yield has been found to be 0.81 using pooled estimates from several authors (Maijala and Hanna, 1974). In practice, fat yield is often estimated as the product of milk yield \times % fat at various stages summed over the lactation, so that the observed correlation is likely to be biased because of the correlated error terms involved. A more meaningful correlation is probably that between milk yield and % fat. Taylor (1973) in discussing the inter-species relationship between milk yield and body weight observed that "the not too far from average milk energy production of cattle is seen to be achieved by producing well above the average quantity of well below average quality milk". Within the bovine species, a pooled estimate of the genetic correlation between milk yield and % fat gave -0.31 (Maijala and Hanna, 1974): other workers more recently found values of -0.54 (Syrstad, 1975) and +0.12 (O'Connor, 1976). The correlation of milk yield and % protein is of similar magnitude. Selection for milk yield alone could therefore affect milk quality. In the last year or two in the U.K. selection of dairy bulls has been performed on the basis of total solids yield. This trait is more likely to be positively correlated with % solids.

Growth rate and body size.

In the U.K. the dairy herd is an important source of meat. Some 40-50% of cattle slaughtered for beef are of pure dairy breeding (Preston and Willis,

1970) while as much as two-thirds of home-produced beef carries some Friesian blood (O'Connor, 1978). Many authors have suggested that milk yield and body weight in the same animal are positively correlated (see Preston and Willis, 1970; Taylor, 1973), especially on high levels of feeding (Robertson, 1973). This encourages the belief that milk production may be correlated with meat production in male relatives. The balance of the evidence suggested that this relationship is either zero or slightly positive (Preston and Willis, 1970). Recently Cunningham, O'Byrne and Mescal (1976) compared the results of beef and dairy progeny tests for 96 Friesian bulls in Ireland on the basis of 1000 beef progeny and 6000 dairy progeny. They found a significant correlation between breeding values for milk yield and for carcass weight for age ($r = 0.47$; 95% conf. int. 0.09-0.82). Moreover, the possible relationship of milk production to carcass fatness or leanness cannot be ruled out. Evidently the links between the physiology of growth and that of lactation require clarification.

Other effects

Taylor, Monteiro, Murray and Osmond (1972) suggested that a dairy bull's breeding value may be negatively correlated with his "mate effect"; that is, a bull expected to give increased milk yield in his daughters may have a small adverse effect on the milk yield of his mates. They propose that this effect is physiologically mediated but there are no other reports to support this.

Possible relationships of milk production and other characteristics e.g. dystocia calving interval, conception rate, production disease, disease resistance, all tend to be studied at the herd level or the population level by statistical methods rather than at the individual level by physiological methods. Both have a contribution to make in clarifying the links between milk yield and other economic traits.

I.2. The Endocrinology of Lactation.

There are three important ways in which hormones may influence the level of milk production. The first is by stimulation of mammary growth prior to lactation, particularly during pregnancy. It might be expected that the larger the mammary gland, the greater would be its milk-producing capacity. Secondly, certain hormones have a known galactopoietic effect and are capable of stimulating milk production and secretion by mammary gland tissue at the cellular level. These effects can be demonstrated by bioassays or in explanted tissue cultures. The third way in which hormones could influence milk production is by hormonal control of energy partition, whereby milk yield may be determined by the amount of energy made available to the mammary gland. These three modes of action are discussed in the following three sections.

I.2.1. Hormones and mammary growth.

During pregnancy, extension and branching occurs of the duct system and vascular elements in the mammary gland. Alveoli are developed, and the lobuloalveolar system progressively takes over most of the space occupied by the stroma (adipose tissue) until the gland becomes a compact mass of lobules of alveoli separated from each other by septa of connective tissue. Cell division occurs throughout pregnancy (Cowie and Tindal, 1971).

In dairy cattle some mammary growth occurs at puberty but the greater part occurs during pregnancy. Since there is little regression of mammary tissue during a normal dry period of two months or less (Swanson, Pardue and Longmire, 1967), it seems likely that mammary growth during the first pregnancy may have a lasting effect on mammary development.

Comparing a wide range of mammalian species, Linzell (1972) found a strong correlation between milk yield and mammary gland weight. He found a relationship between daily milk yield (kg) and empty mammary gland weight (kg) of the form: milk yield = $3.0 \times \text{gland wt.}^{0.93}$ with a correlation $r = 0.99$. This gives some indication, perhaps, of the importance of mammary growth.

The hormones thought to be principally responsible for mammary growth, both during puberty and pregnancy, are oestrogen and progesterone. Lactation can be induced even in non-pregnant heifers by the administration of $17-\beta$ -oestradiol and progesterone (Smith and Schanbacher, 1973). The treatment used was as follows: heifers between 3 and 18 days post oestrus received $17-\beta$ -oestradiol (0.1mg/kg body wt./day) and progesterone (0.25mg/kg body wt./day) administered as twice daily subcutaneous injections for seven days. Milking commenced 21 days after the first injection. Good udder development occurred and nearly normal milk yields were obtained although it took longer than normal for daily yield to reach its peak value. Similar results were achieved in heifers and cows by Collier, Bowman and Hays (1975), who used the same treatment with the addition of single daily intramuscular injections of dexamethasone on days 18, 19 and 20. This gave no substantial improvement. Erb, Malvern, Monk and Mollett (1976) used the same treatment and found that higher levels of plasma oestrogen were associated with the better responses in their group of cows.

In mice, DNA synthesis in the mammary gland was inhibited completely by ovariectomy (Bresciani, 1964). Nevertheless, good udder development was obtained in ovariectomized heifers by injection of $17-\beta$ -oestradiol and progesterone three times weekly (Tucker, 1969). In non-pregnant heifers, mammary gland development based on determinations of DNA, RNA, collagen and lipid is

greater on the day of oestrus, when oestrogen levels are high, than at other times in the oestrous cycle (Sinha and Tucker, 1969).

There is some evidence (Auran, 1974; Syrstad, 1974; Wood, 1975) that increased milk production occurs in lactations initiated by multiple pregnancies when there are two placentae and, in most cases, two corpora lutea contributing to secretion of these hormones. There is also evidence that dams carrying larger calves may show higher production in the subsequent lactation, since oestrogen levels in late pregnancy were highly correlated with the birth weights ($r = 0.84$) and weaning weights ($r = 0.87$) of the calf or calves born to Charolais cows (Terqui, Delouis, Thimonier and Ortavant, 1975). Similarly, cows induced into parturition had significantly lower oestrogen levels, had smaller calves and gave a below-average milk yield (Beardsley, Muller, Garverick, Ludens and Tucker, 1976).

Besides oestrogen and progesterone, another placental hormone, bovine placental lactogen, has been shown to be associated with milk yield (Bolander, Ulberg and Fellows, 1976). Measurements of this hormone during the last three months of pregnancy showed higher levels in dairy than in beef cows, while within the dairy breed placental lactogen levels were correlated with subsequent milk yield.

There may exist an effect of a dairy bull on his mate's milk yield, that is, an effect of the sire of the calf on the ensuing lactational performance of the cow (Skjervold and Finland, 1975; Taylor et al., 1979). Such an effect could be mediated by the inherited hormone-secreting ability of the foeto-placental unit.

The overall picture, then, is that secretion of hormones by the placenta and the maternal ovary influence mammary growth which may subsequently have some effect on milk production. However, this physiological knowledge offers little opportunity for improving milk yield. Selection for udder size would hold few advantages since most mammary growth occurs during pregnancy and milk yield itself is measurable shortly afterwards. Allaire et al. (1973) found a significant correlation ($r = 0.5$) between oestrogen levels and breeding value in prepubertal Holstein calves. Oestrogen was measured in 54 heifer calves in two herds. Breeding value for each calf was taken as a combination of the sire's breeding value and the dam's first lactation record. When the oestrogen levels were adjusted for age, the regression of breeding value on oestrogen level was significant. However, no new information on this work has emerged. Finally, it may be possible to identify certain bulls whose fetuses stimulate mammary growth during pregnancy more than those of other bulls (Skjervold and Finland, 1975; Taylor et al., 1979). Consideration of this effect when practising bull selection might generate small improvements in cows' yields.

I.2.2. Hormones and lactogenesis.

Lactose synthesis from glucose occurs exclusively in the mammary gland and is catalysed by 5 enzymes: hexokinase, phosphoglucomutase, UDP-glucose pyrophosphorylase, UDP-glucose-4-epimerase and lactose synthetase. Each of these enzymes is present from 30 days prepartum in bovine mammary tissue, and the capacity of the tissue to produce lactose increases about 1.5 to 2.5 times from late gestation to early lactation (see Convey, 1974).

Lactogenesis also demands the capacity to synthesize fatty acids, since 60% by moles of the fatty acids in ruminant milk are synthesized from acetate or

β -hydroxybutyrate by the mammary gland (Palmquist, Davis, Brown and Sachan, 1969). Two key enzymes in this pathway are acetyl-CoA carboxylase and acetyl-CoA synthetase, both of which show considerable increase around parturition; so also does lipoprotein lipase which is required to hydrolyse triglycerides in the plasma for uptake by the mammary gland (Shirley, Morrow and Emery, 1973).

It is clear, therefore, that much of the mammary preparation for milk production occurs before parturition. At parturition, various hormones interact to activate these enzyme systems and milk secretion commences. The relevance to lactation of the various endocrine changes around parturition is difficult to assess because of the stress occurring at this time, the metabolic demands for energy and protein, and the complexity of the hormonal control of parturition itself. Thus, although the initiation of lactation has been widely studied, the hormonal mechanisms whereby it is achieved are not fully understood.

Levels of oestradiol, oestrone and total oestrogens increase markedly in the month preceding parturition. This increase coincides with the increased enzyme levels in the mammary gland, but no causal relationship has been established. Placental lactogen follows a roughly similar pattern, rising in late pregnancy. Besides their possible effects on mammary growth and enzyme activity, it is not thought that any of these hormones is responsible for the initiation of lactation as their levels fall at partition.

Although progesterone is necessary for normal mammary development in induction treatment, some authors feel that progesterone is responsible for the repression of lactation during pregnancy, and that the precipitous decrease in progesterone levels prepartum allows activity of the mammary gland to begin. One means by

which this could occur is that progesterone is known to inhibit synthesis of α -lactalbumin (Palmiter, 1969). Normally lactose synthetase does not accept glucose as a substrate; however, α -lactalbumin alters the specificity of the enzyme to include glucose and so enable lactose synthesis. The drop in progesterone level prepartum may allow synthesis of α -lactalbumin and consequent lactose synthesis and milk secretion. Milk protein also contains α -lactalbumin (Linzell and Peaker, 1971).

Another way in which progesterone may be connected with the initiation of lactation is through prolactin. Cessation of progesterone infusion is known to trigger prolactin secretion (Karg and Schams, 1974) and a large prolactin peak occurs at the time of parturition which is thought to play an important part in initiating lactation. Peaks of adrenocorticotropin (ACTH) and corticosteroids also appear at parturition. Mammary explants from pregnant mice require prolactin and cortisol to commence synthesis of milk proteins (Voytovich, Owens and Topper, 1969). An experiment performed on one cow suggests that abolition of the prolactin peak using the blocking agent 2-bromo-ergocryptine, or CB154, adversely affects milk production in the subsequent lactation since the cow almost failed to lactate (Schams, Reinhardt and Karg, 1972). However, the corticosteroid peak may also be involved, since injection of exogenous corticosteroids during early pregnancy causes milk secretion in heifers (Tucker and Meites, 1965). Treatment of two cows with thyrotropin releasing hormone (TRH) from 15 days prepartum to 5 days postpartum gave an increase in milk yield of about 40% relative to their identical twins (Karg and Schams, 1974). This effect may have been mediated by prolactin since TRH stimulates prolactin secretion (Convey, Tucker, Smith and Zolman, 1973), or may alternatively have been mediated by the resulting higher expected levels of thyroxine (for beneficial effects of thyroxine on milk yield see next section).

The release of anterior pituitary hormones is now thought to be stimulated by releasing factors secreted by the hypothalamus. The tripeptide TRH causes release of thyroid stimulating hormone (TSH) in cattle (Kelly, Bodirian, Baker and Friesen, 1973) and secondarily increases serum thyroxine and triiodothyronine (Convey, Chapin, Kesner, Hillman and Curtis, 1977). TRH also increases concentrations of prolactin and growth hormone (Convey *et al.*, 1973). The decapeptide luteinising hormone release hormone (LHRH) causes release of LH and follicle stimulating hormone (FSH) (Schally, Arimura, Baba, Nair, Matsuo, Redding, Debeljuk and White, 1971). Recently other factors have been discovered which stimulate growth hormone secretion (Nair, De Villier, Barnes, Antalis and Wilbur, 1978) and ACTH secretion (Vale, Rivier, Young Minick and Guillemin, 1978). These releasing factors appear to cause depolarization of the trophic cell membrane and subsequent hormone release.

Prolactin-secreting cells appear to be atypical in that they depolarize spontaneously and continue to release prolactin unless hyperpolarized, and so inhibited, by the hypothalamic prolactin inhibiting factor (PIF). Prolactin secretion from the anterior pituitary is inhibited by the injection of hypothalamic extract, but severance of the pituitary stalk allows secretion to continue for over 30 days (Meites, 1966). Although hypothalamic stimulation depresses prolactin release, the suckling stimulus causes marked surges in prolactin. The amount released, as measured by serum levels 5 minutes after milking, appears to parallel the level of milk production ($r = 0.6$) and decreases as lactation progresses, whereas the 'basal' levels of prolactin, as measured 4 hours before milking, gradually rise over the lactation (Koprowski and Tucker, 1973). It has been suggested that these surges of prolactin on suckling were responsible for the maintenance of lactation, but suppression of prolactin using CB154 does not seem to affect milk production (Karg *et al.*, 1972). These

prolactin surges on suckling do not therefore seem to have the same importance in maintaining lactation that the peripartum prolactin peak has in initiating it (see above). Kahn and Martinet (1975) abolished the prolactin surges after suckling in sheep by severing the nervous connections between the mammary gland and the body. This procedure did not affect basal levels of prolactin. Milk production, as assessed by rate of lamb growth, was normal but the ewes returned to oestrus earlier than controls.

Hart, Bines, Balch and Cowie (1975) found significantly higher prolactin concentrations in lactating beef cows giving poor yields of milk than in dairy cows giving good yields. This finding seems to agree with that of Koprowski and Tucker (see above) where basal prolactin levels were low at peak lactation and increased as lactation progressed.

Other pituitary hormones tend to show higher levels when lactation is more intense. Levels of growth hormone are elevated during early lactation and decrease as lactation advances (Koprowski and Tucker, 1973) and are significantly higher in lactating dairy cows than beef cows (Hart et al., 1975). This is in accord with the known galactopoietic effect of growth hormone which is thought to act by stimulating the synthetic activity of alveolar cells (Cowie and Tindal, 1971). Levels of ACTH are also known to be higher at peak lactation (Voogt, Sar and Meites, 1969). Convey et al., (1977) found no change in levels of TSH or magnitude of TRH-stimulated TSH release with stage of lactation, but thyroxine levels are known to be depressed during early lactation and rise towards the end of lactation (Lorscheider, Oxender and Reineke, 1969; Hart et al. 1978). This would suggest that TSH levels might fall due to negative feedback. Levels of LH and FSH during lactation do not seem to have been studied.

The above evidence suggests an important role for the hypothalamus during lactation and possibly in initiating lactation. It seems possible that activation of the trophic hormone releasing factors in the basal hypothalamus is brought about by some metabolic means analogous to the control of the 'hunger centre' in the lateral hypothalamus (Cowie and Tindal, 1971). At the height of lactation it seems that the releasing factors stimulate the pituitary to produce elevated levels of GH and ACTH and depressed basal levels of prolactin, together with above-average surges of prolactin in response to suckling.

Of the studies discussed above, only that of Hart et al. (1975) involved the comparison of cows at the same stage of lactation producing different amounts of milk. Even in that study, however, the observed hormone differences could be interpreted either as being causative, i.e. controlling the milk yields, or as being secondary, i.e. resulting from the different states of energy balance which the two groups of cows had attained (see next section). It is difficult, therefore, to draw conclusions about which hormones might have differential effects on milk secretion in genetically different animals. Tucker, Koprowski, Britt and Oxender (1973, 1974) measured levels of prolactin and growth hormone in dairy cows and bulls. They found low repeatabilities, near zero heritabilities and no correlation of either hormone with breeding value for milk production. They concluded that: "simple basal estimates of these hormones will have no usefulness in selection processes for dairy cattle until sampling variation is more completely defined or controlled. Estimates of hormones after application of suitable stimuli may be more closely related to milk production than relationships in this paper". It is possible that observed hormonal effects on milk secretion may only be observable during the lactation itself. In this case it may indeed be true that "application of suitable stimuli" might offer

the most fruitful approach. In this way it is possible to test, for instance, the hypothalamic sensitivity even of males and non-lactating females. The pattern of their hormonal response might be related to hormone secretion during lactation and hence to milk production.

I.2.3. Hormones in relation to energy partition

Increasing milk energy output

In order to increase milk energy output per cow, it is necessary to achieve one or more of the following: (i) increase the metabolizable energy (ME) "throughput" (ii) increase the efficiency of utilization of ME, i.e. increase the proportion of ME intake used for anabolic processes and reduce the proportion lost as heat, urine, etc. (iii) increase the proportion of ME utilized for milk production rather than other anabolic processes, particularly body tissue deposition; this last possibility also requires increased throughput on a lifetime basis if a constant quantity of energy is made available for the anabolic processes other than milk production. Increased energy input alone can have only limited success. Nutritional studies have shown that each succeeding unit of feed input results in successively smaller increments of milk output and larger increments of body gain, although individual cows do vary in the amount of milk produced before substantial amounts of dietary energy are diverted to form body tissue. It follows that even if it is possible to select for increased intake, it is still necessary to understand the hormonal control of metabolic processes so that the extra metabolite supply is partitioned into the desired pools.

"The efficiency of metabolizable energy utilization computed by linear regression analysis indicated that $65.8\% \pm 1.8\%$ of the ME was secreted as milk energy and deposited as body tissue. Differences in the efficiency of conversion ... due to level of milk production, amount of body tissue deposition (or loss) and stage of lactation were minor. The major difference between rations as well as between animals was in the amount consumed and where the energy was used (i.e. milk production or fattening) rather than the efficiency with which it was used," (Flatt, Moe, Munson and Cooper, 1969). On a particular ration and under set conditions, there seems to be little variation among animals in the amount of energy lost as urine, faeces and heat production combined; there is correspondingly little variation in the efficiency of utilization.

It seems likely, then, that increased milk energy output is most likely to be achieved by increasing the proportion of ME utilized for milk production as opposed to body tissue deposition. Broster, Broster and Smith (1969) conducted extensive nutritional investigations into lactating dairy cattle and showed that high- and low-yielding cows differed chiefly in the proportions of dietary energy they diverted to milk or to body tissue. Others have noted the capability of high producing cows to mobilize large quantities of body energy during early lactation and to deposit it during late lactation (Flatt et al., 1969; J. Hodges, personal communication).

To a small extent, the composition of the ration can influence the proportion of ME diverted to milk. For instance, increasing the proportion of concentrates relative to forage tends to raise the ratio of propionate : acetate among the volatile fatty acids in the rumen; this in turn leads to increased fat deposition and reduced milk energy output (Flatt, Moe, Moore, Lehmann, Orskov

and Hemken, 1969; Platt et al., 1969). Similarly, ruminal infusion of acetate in cattle to supplement a basal diet produces larger increments in milk production than infusion of propionate which results more in deposition of body tissue (Ørskov, Platt, Moe, Munro, Hemken and Katz, 1969).

Overall, however, it seems likely that genetic variations in the partition of nutrients towards milk or meat are brought about chiefly by inherited patterns of endocrine function. Several hormones have been studied in relation to energy partition during growth, fattening and lactation. This work is described in the remainder of this section.

Hormones in relation to growth, fatness and milk yield.

Growth hormone (GH) is secreted by the anterior pituitary and has a molecular weight of about 22,000. It has two main effects, an anabolic effect whereby it promotes protein deposition and muscle growth, and a lipolytic effect whereby it stimulates fat mobilization from body tissue (Tanner, 1972). GH is secreted in a pulsatile manner and in general exhibits a fairly low repeatability of measurement over time (Bassett, 1974), as do many pituitary hormones.

Consequently, some of the clearest results have been obtained by administration of exogenous GH. In pigs, whether on ad libitum or restricted feeding, the administration of 0.13 mg porcine GH per kg body wt. per day increased daily gain, percent lean, and food conversion efficiency, and decreased backfat thickness relative to controls (Machlin, 1972). Growth hormone secretion was not stimulated by reduced glucose levels or increased levels of acetate, propionate or butyrate (McAfee and Trenkle, 1971), nor by feeding (Hove and Blom, 1973), but is stimulated by reduced levels of free fatty acids (Reynaert, De Paepe, Marcus and Peeters, 1975; Quabbe, Ramek and Luyckx, 1977).

During lactation, exogenous GH increased milk production by 18% (Machlin, 1973). Although GH is known to have a direct galactopoietic effect on the mammary gland (Cowie and Tindal, 1971), at least some of this effect is probably due to the mobilization of adipose tissue reserves for lactation. Additionally, GH may increase the rate of gluconeogenesis. Hart et al. (1975) studied three lactating beef (Hereford cross) and three dairy cows (Friesian) which had been matched for age, stage of lactation and diet. During the first 15 weeks of lactation the beef cows gained weight and gave a poor yield of milk, whereas the dairy animals gave higher yields but lost weight. They observed significant differences between the two groups in the levels of several hormones, and GH was much higher in the dairy cows (4.2 v 1.5 ng/ml; $P < 0.005$). This difference is obviously related to the difference in energy partition. Tucker et al. (1974) measured GH in Holstein bulls and found no correlation of GH levels with breeding value for milk yield. However, the repeatability of measurement was only 0.30 and the heritability not different from zero.

Insulin is a polypeptide hormone of molecular weight about 8000 secreted by the pancreas. Insulin has many functions, its basic effects being to increase energy retention by body tissues and reduce the amounts of energy metabolites circulating in the blood. Known effects of insulin include 1) inhibition of glycogenolysis 2) inhibition of gluconeogenesis 3) inhibition of lipolysis 4) stimulation of lipogenesis 5) inhibition of proteolysis (Sonksen and Brown, 1977). In non-ruminants, glucose entry into the bloodstream is thought to be an important stimulator of insulin secretion. Insulin is important in removing glucose from the blood and for transporting it into cells; deficiency results in diabetes. In ruminants, however, little glucose is absorbed from the gut and most is synthesized de novo from volatile fatty acid precursors : about 65-85% of glucose is synthesized from propionate (Thatcher, Herbein, Schmidt, McGowan,

McGilliard and Young, 1977). Although glucose infusion triggers an insulin response in ruminants, infusion of propionate or butyrate elicits a much larger response (McAtee and Trenkle, 1971; Ambo, Takahashi and Tsuda, 1973). In non-ruminants glucose elicits a large insulin response whereas propionate has no effect (Horino, Machlin, Hertelendy and Kiphis, 1968).

Food intake also stimulates insulin release. In ruminants there appears to be an initial short-term response, followed by a delay of 1-2 hrs in lambs, 2-3 hrs in adult sheep (Bassett, 1974) and 3-6 hrs in cattle (Hove and Blom, 1973) before the second and larger response. This second response is thought to occur as ruminal fermentation proceeds and nutrients begin to be absorbed from the gut. Much of the observed variation in insulin levels appears to be prandial (Hove, 1974; Trenkle, 1978). Even in ruminants, insulin levels are correlated with levels of circulating glucose : correlations of 0.64 for sheep on ad libitum feeding (Bassett, 1974) and 0.45 for cows (Hove, 1974) have been found. Glucose disposal is determined primarily by insulin rather than any other hormone (Felig, Wahren, Sherwin and Hendler, 1976).

Insulin also plays an important part in regulation of lipid metabolism. Nearly all forms of genetically transmitted obesity in rodents are accompanied by abnormal elevation of insulin levels (Bray and York, 1971). Gregory, Wood and Lister (1976) studied the relationship of insulin to fatness in Pietrain and Large White pigs. Using the ratio of subcutaneous fat wt. : longissimus dorsi muscle wt. as a measure of fatness, they found a correlation of 0.98 between this character and fasting insulin levels.

Addition of starch to the diet causes increased nitrogen retention, and presumably reduced protein breakdown in pigs. This may be mediated by insulin.

Fuller, Weekes, Cadenhead and Bruce (1977) infused insulin and glucose into pigs and found a significant reduction in plasma urea levels. Infusion of insulin alone would probably have induced hypoglycaemia with secondary rises in glucagon and corticosteroids which stimulate proteolysis.

The effects of insulin in protein metabolism are to some extent synergistic with those of growth hormone so that both are required for the full anabolic effect (Daughaday, Herington and Phillips, 1975). In its antilipolytic effect, however, its action is antagonistic to that of GH. Contrary to the effect of GH, injection of exogenous insulin during lactation depresses milk yield in cattle, probably by reducing the level of circulating glucose (Kronfeld, Mayer, Robertson and Raggi, 1963). Koprowski and Tucker (1973) found a significant negative correlation between insulin levels and milk yield ($r = -0.3$, $P < 0.01$). In the experiment described above, Hart et al. (1975) found that in early lactation the mean insulin levels over 24 hours were 9.5 and 28.8 $\mu\text{U/ml}$ respectively for the dairy and beef cows ($P < 0.001$). Since food intake was the same for both groups, it seems likely that the different insulin levels were related directly to the differences in energy partition. Hove (1974) observed a significant fall in insulin levels after the onset of lactation, and suggested that the decreased levels were important in facilitating lipolysis (fat mobilization) during hypoglycaemia.

Thyroxine (T_4) and triiodothyronine (T_3) are the major thyroid hormones and exert a profound effect on energy metabolism. Their main effect is to stimulate basal metabolic rate and so increase heat production. T_3 is 2-4 times more active than T_4 in this respect in man, but concentrations of T_4 are about 50-fold higher than those of T_3 (Chopra, Carlson and Solomon, 1978). Production and release of thyroid hormones is controlled by the anterior pituitary hormone TSH which in

turn is dependent upon the positive stimulus of TRH and the negative feedback of the thyroid hormones.

Ablation of the thyroid depresses growth in cattle (Blaxter, Reineke, Crampton and Peterson, 1949). T₄ levels were correlated with growth rate in beef bulls ($r = 0.2-0.3$; Bard, 1973). Pietrains, a lean breed of pig, were found to have a higher turnover of thyroid hormones than the fatter Large Whites, but little difference in overall thyroid status was observed (Lister, 1976). Daily injection of TRH into calves for 90 days increased weight gain by 10% but food efficiency remained constant (McGuffey, Thomas and Convey, 1977). This effect may have been mediated by elevated levels of T₄ or GH. Levels of T₃ are correlated with weight gain in man (Danforth, 1975).

Levels of T₄ are slightly reduced and levels of T₃ significantly reduced during fasting (Carlson, Drenick, Chopra and Hershmann, 1977), or in anorexia nervosa in man (Croxson and Ibbertson, 1977) but increase on re-feeding (Spaulding, Chopra, Sherwin and Lyall, 1976). This is probably due partly to reduced secretion and partly to reduced formation of T₃ by deiodination of T₄ in peripheral tissue. The overall effect is to reduce metabolic rate and conserve energy in a time of caloric deprivation.

There appears to be a similar response during lactation. Lorscheider et al. (1969) found that T₄ levels at peak lactation were about 54% of those observed in the dry period. Hart et al. (1978) also found reduced T₄ levels during lactation relative to the dry period but found no differences in T₄ levels between high-yielding (dairy) cows and low-yielding (beef cows). On the other hand, Vanjonack, Bode and Johnson (1977) found a negative correlation between plasma T₄ level and milk yield ($r = -0.50$; $P < 0.01$).

Dairy cows were found to have larger thyroids than beef cows (Blaxter et al., 1949). There is also extensive evidence from early work that exogenous thyroxine or thyroprotein has a beneficial effect on milk production (see reviews by Meites, 1961; Cowie, 1966). Oral administration of these substances increased milk yields by about 25%, although food intake also increased and the relative conversion efficiencies before and after treatment have not been investigated. Karg and Schams (1974) have shown a 40% increase in the yield of two cows relative to their identical twins following TRH administration over a period of 20 days prior to the start of lactation. This may have been due to resulting elevated levels of T₄, or alternatively to raised levels of prolactin (see above).

Other workers have reported a genetic relationship between thyroid activity and milk production. Joakimsen, Steenberg, Lien and Theodorsen (1971, 1975) evaluated 97 mature bulls and found correlations between 0.14 and 0.47, depending on breed, between breeding value for fat-corrected milk and thyroxine degradation rate (TDR). TDR was estimated as the product of plasma thyroxine level, thyroxine distribution value and disappearance rate of radioactive thyroxine from plasma (Yousef and Johnson, 1967). Repeated measurements were performed on each bull and over several experimental groups the intraclass correlation between measurements within bulls ranged from 0.35 to 0.87. The estimated genetic correlation of TDR with breeding value for milk production lay between 0.22 and 0.57. In a second investigation Joakimsen (1975) reported TDR and serum triiodothyronine levels in 94 young bulls. Their results are set out in the accompanying table. The correlation between thyroxine level and milk yield is not reported.

Table I.1. Estimated correlation between thyroid activity measured on young bulls and milk yield of their daughters.

From Joakimsen (1975), Table 2.

No. of bulls	Age at hormone test	Type of hormone test	Average No. of daughters per sire	Milk production characteristics	
				Maximum daily yield	Lactation yield
	(Mo)			—	r —
80	4-12	Thyroxine degradation rate	125	0.12	0.16
		Plasma level of triiodo-thyronine		0.25*	0.03
14	12-16	Thyroxine degradation rate	262	0.40	0.25
		Plasma level of triiodo-thyronine		0.05	-0.09

* P 0.05

As indicated in the table, TDR show a moderate, but consistent positive correlation with total milk yield. On the contrary, the triiodothyronine level shows no fixed relationship to milk yield.

For selection purposes the product of the genetic correlation and the accuracy of the thyroid activity estimates is most important. Joakimsen states that the expectation of this product lies between 0.2 and 0.5. However, the method used for estimating TDR may not give a true assessment of thyroid status. The method involved the assumption of single compartmental kinetics, i.e. that no significant error is introduced by treating the system as a single homogeneous

rapidly equilibrating compartment into which and from which thyroxine is distributed at a constant rate. The validity of this method of analysis has been questioned (Oppenheimer, Schwartz and Surks, 1975). Furthermore, the technique used involves the injection of radioactive thyroxine. Graf and Krausslich (1975), in reviewing the work of Bard (1973) and Joakimsen concluded that the use of a measure of thyroid activity as a selection criterion "cannot be recommended at this time" since 1) estimates of thyroid activity were probably not reflecting thyroid status sufficiently accurately 2) observed correlations between thyroid activity and performance traits did not necessarily mean that the relationship was linear 3) altered thyroid status might affect energy metabolism, maintenance requirements or efficiency; but they found the correlation interesting and recommended further study.

Of other hormones which might be thought to influence energy metabolism, the glucocorticosteroids show little variation in their basal levels and are uncorrelated with milk production (Koprowski and Tucker, 1973). However, surges of corticosteroids, like prolactin, occur in response to suckling and these surges may be more relevant to milk yield. It might be thought that corticosteroids would stimulate milk production by increasing catabolism of body protein and supplying more glucose to the mammary gland, but injection of exogenous corticosteroids depresses milk production in the cow (Campbell, Davey, McDowell, Wilson and Minford, 1964). Glucagon levels might also be expected to affect milk yield since glucagon stimulates gluconeogenesis, but this hormone has apparently not been studied in connection with lactation in cattle despite its probable importance.

Hart et al. (1975) compared hormone and metabolite levels in lactating beef and dairy cows as described above. They extended this comparison by considering the ratios of concentrations of several hormones to each other and to those of metabolites (Hart et al., 1978). When this was done, certain differences were even more pronounced. Other workers have measured the concentrations of certain blood constituents in dairy cows (Rowlands and Manston, 1976), dairy heifers (Kitchenham and Rowlands, 1976) and dairy bulls (Stark, Rowlands, Manston and McClintock, 1978). In heifers they found correlations between milk production and levels of urea ($r = 0.39$) and inorganic phosphate ($r = -0.39$) in the blood, while in bulls they found correlations between breeding value and levels of urea ($r = 0.24$), inorganic phosphate ($r = -0.24$) and potassium ($r = -0.25$). These results suggest that bulls of differing genetic merit do differ in some aspects of their physiological processes, and it is possible that observed differences among them are related to and controlled by hormone levels.

In conclusion, it seems likely that some of the variation in lactation yields in cattle may be ascribed to differences in energy partition between milk and other pools, and that this partition may be under endocrine control. Identification of different patterns of hormone secretion in groups of animals known to differ genetically in their merit for milk production may enable selection for improved lactation performance.

I.3. Aims of study.

Three ways have been considered in which hormone levels might act to influence the level of milk production in dairy cattle : by promoting mammary growth, by stimulating the mammary gland to secrete milk and by partitioning a greater proportion of dietary energy towards milk production. On the basis of the evidence given above, it seems that the third possibility offers the greatest opportunity for creating physiological aids to selection for milk production. It seems likely that genetically different cows exhibit different patterns of hormone secretion during lactation which divert greater or lesser amounts of energy towards milk production. It is possible that the levels of these hormones or their patterns of secretion might show corresponding differences among animals even when not lactating. It might then be possible to identify superior dairy animals at an early age.

As was shown in the previous section, insulin, growth hormone and the thyroid hormones seem to play an important part in controlling the energetics of milk production. It was decided to measure insulin, T₄ and T₃ in animals of known differing genetic potential for milk production at various stages in the life cycle. Growth hormone was also measured in experiment 3 by Dr. I. C. Hart.

The aims of the study were : 1) to clarify the predictive value of these hormones for milk production 2) to estimate their repeatabilities 3) to ascertain further their relationships with each other and with other traits 4) to ascribe variation to possible sources other than milk production such as breed, age, sex, seasonal, diurnal and prandial variation.

CHAPTER II : ASSAY METHODS.

II.1 Thyroxine solid phase radioimmunoassay.

II.1.1 Principle.

The method used for thyroxine assay was that of Seth, Rutherford and McKenzie, 1975. Thyroxine levels in plasma were determined by radioimmunoassay using thyroxine antibodies which have been made insoluble by covalent coupling with microcrystalline cellulose. Plasma samples were added directly to the assay incubation mixture, interference in the antigen/antibody reaction by the thyroxine binding serum proteins being almost completely eliminated by the addition of 8-anilino-1-naphthalene sulfonic acid (ANS) and incubation at pH 10.5. Residual interference was compensated for by preparation of standards in thyroxine-free serum. Use of antibodies in this solid-coupled form permits separation of free and antibody-bound hormone by a single washing step followed by centrifugation and decantation of the supernatant to waste. All dispensing and dilution of samples and reagents was done by an automatic sample processing unit. The precipitate was counted by an automatic gamma counter with a paper tape output; the data from the tape were analysed by computer using a radioimmunoassay programme for statistical analysis of the assay and estimation of hormone levels for the unknown samples.

II.1.2 Materials.

The thyroxine antiserum was raised in sheep and was kindly supplied by Dr. J. Seth, Royal Infirmary, Edinburgh. The antiserum was coupled to

activated cellulose by the following procedure. 500 μ l of antiserum and 1.0g of cyanogen bromide-activated cellulose were added to 2.0ml of borate buffer (0.1 mol/l, pH 8.6). The suspension was mixed in a rotating mixer for 3 days at 4 $^{\circ}$ C. The coupled antiserum was then washed three times in the borate buffer, twice in acetate buffer (0.1 mol/l, pH 4.0), and three times in the assay buffer. The duration of each wash was 30 minutes at 4 $^{\circ}$ C. Coupled antiserum was stored at a 100-fold dilution in assay buffer at 4 $^{\circ}$ C.

The assay buffer was sodium glycinate (0.1 mol/l, pH 10.5) containing gelatin (2g/l) and sodium azide (100mg/l).

3', 5' - 125 I-L-thyroxine, specific activity 50 μ Ci/ μ g, was obtained from the Radiochemical Centre, Amersham. The working solution of ANS/tracer was prepared by adding 100 μ l of 125 I thyroxine and 500 mg ANS (Sigma) to 400 mls of glycinate buffer. This solution was stored at 4 $^{\circ}$ C.

Thyroxine-free serum was prepared by treating 500 mls of bovine serum with 100g activated charcoal and mixing for 24 hrs at 4 $^{\circ}$ C. Extraction was followed by ultra-centrifugation and filtration through a 3 μ m Millipore filter. The filtrate was stored frozen until used for preparation of standards.

L-thyroxine was obtained from Sigma Chemical Co. The thyroxine stock standard was prepared by dissolving 18.0 mg L-thyroxine sodium salt pentahydrate in propylene glycol/water (1:1 by volume) adjusted to pH 9.0 with NaOH. Working standards for use in the assay were prepared by serial dilution of this stock standard in T4-free serum.

The wash solution used was 0.1 mol/l NaCl containing 0.06% Decon.

The sample cups were polystyrene 3 ml cups (Sarstedt). The assay incubation was performed in polystyrene round-bottomed tubes (Luckhams LP4's). Initially polystyrene tubes from Nunc UK were used. However, the second batch of these tubes was found to give very high (10%) non-specific binding of labelled hormone in several different hormone assays so that spurious values were obtained for the blank tubes. After this was discovered, the Luckhams tubes were used.

II.1.3 Assay Method.

Using the Analmatic sample preparation unit (Searle Instruments) 2 replicates of 25 μ l of either standard or unknown plasma were picked up and dispensed into the reaction tubes, each washed through by a 200 μ l dispense of the ANS/tracer solution. At the same time, 500 μ l of the solid-coupled antibody were dispensed from a stirred suspension in glycinate buffer to give a final antibody dilution of 4500- to 6000-fold in an incubation volume of 725 μ l. Thus each incubation mixture for the standards contained 0.5 to 6.25 ng T₄ in 25 μ l T₄-free serum (equivalent to 29, 40, 60, 80, 100, 200 and 250 ng/ml) while each tube for the unknown contained 25 μ l plasma. Each standard was performed in quadruplicate, each unknown in duplicate. In addition, all tubes contained 0.2 ng (= 10nCi) ¹²⁵I-T₄ and 250ug ANS. After mixing, the incubation mixtures were stored at 4° C for 24 h. 1 ml of wash solution was then added to each tube and the tubes were centrifuged at 1200g for 30 min. The supernatant was decanted, any remaining droplets being aspirated from the rim of the tube. The amount of antibody-bound tracer in the precipitate was counted on the automatic gamma-counter for 100 seconds.

II.1.4 Assay optimization.

A. Serum protein interference :

A potential problem in radioimmunoassay of plasma T₄ is that serum proteins, especially thyroxine-binding globulin, bind T₄ strongly. In many usual assay diluents these proteins compete with the antibody for binding of T₄ and incorrectly high results are obtained. In the present assay, possible interference is reduced by three means :-

- (i) Use of a high pH in the reaction mixture. Seth et al. (1975) found that higher pH's reduced interference from binding proteins without any reduction in antibody binding up to pH 11.0. Sodium glycinate buffer pH 10.5 was therefore used.
- (ii) Addition of ANS to inhibit T₄-binding by serum proteins without significantly inhibiting T₄-binding by the antibody. Seth et al. (1975) found maximum inhibition of serum proteins was achieved by 0.63 μ mol/tube with greater ANS concentrations causing reduced antibody-binding but no further reduction in interference. These tests were performed in sodium phosphate buffer (pH 7.5). In the present assay, using bovine serum and in glycinate buffer (pH 10.5), greatest inhibition of serum proteins was found at 250 μ g (= 0.79 μ mol) per incubation mixture. The ANS/tracer solution was made up accordingly for each assay.
- (iii) Preparation of standards in T₄-free serum to equalize protein concentrations of standards and unknowns. Under these conditions parallelism was achieved routinely between the dose response curves of the standard solutions and different dilutions of control serum.

B. Antibody dilution and sensitivity :

Solid-coupled antibody was serially diluted to give working dilutions from

1 in 500 to 1 in 64000. For each dilution the percentage of total $^{125}\text{I-T}_4$ bound by antibody for the zero standard (25 μl T_4 -free serum added) was compared with the percentage bound for the top standard (25 μl of serum added containing 6.25 ng, i.e. 250 ng/ml). The result is shown graphically (see Fig. 2.1). The dilution where the greatest difference occurs between the two levels of binding indicates the optimum sensitivity : this is seen to be at antibody dilutions from 1 in 2000 to 1 in 8000, with zero-standard percentage bound at about 35 to 75%. Before each assay the antibody was titrated out to give binding within this range while conserving the stock where possible.

C. Tracer dilution and sensitivity :

Tests were carried out to optimise tracer concentration by running standard curves at 4 different concentrations of $^{125}\text{I-T}_4$. The result is shown graphically (see Fig. 2.2). The optimum concentration appeared to be about 200 pg/tube. Using the tracer at this concentration gave 15,000 to 20,000 counts per 100 seconds. No tracer was used which was more than 4 weeks old.

II.1.5 Sensitivity.

The sensitivity or detection limit, that is the hormone concentration corresponding to a level two standard deviations away from the zero standard, was less than 10 ng/ml in every assay and normally fell between 8-10 ng/ml. Details of the standard curve and quality control will be found in subsequent section II.4 on statistical analysis of assays.

II.1.6 Specificity.

After tests on the antiserum for cross-reaction with a variety of iodinated compounds Seth et al. (1975) conclude : "Our antiserum showed a highly

satisfactory degree of specificity significant interference from tetra-iodoacetic acid is unlikely in view of its low concentration in serum (Burger, Schilter and Sakaloff, 1974). Triiodothyronine in serum, even at the concentrations found in "T₃-toxicosis", would not interfere".

In the present assay, no significant depression of binding was obtained by addition of 50 μ l of serum containing 10 ng/ml of T₃.

Figure 2.1 :

T₄ assay optimization : graph showing assay sensitivity at different dilutions of antibody.

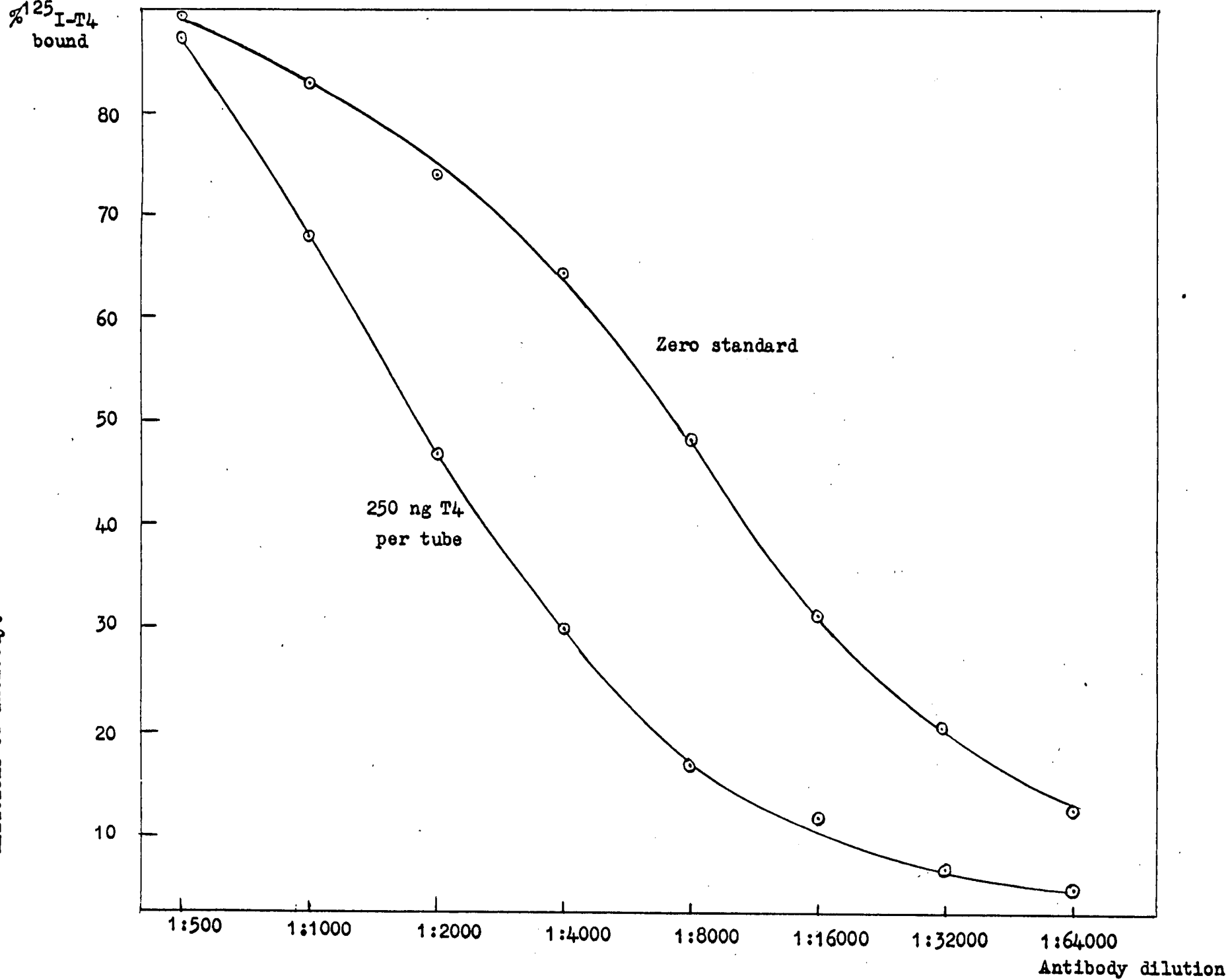
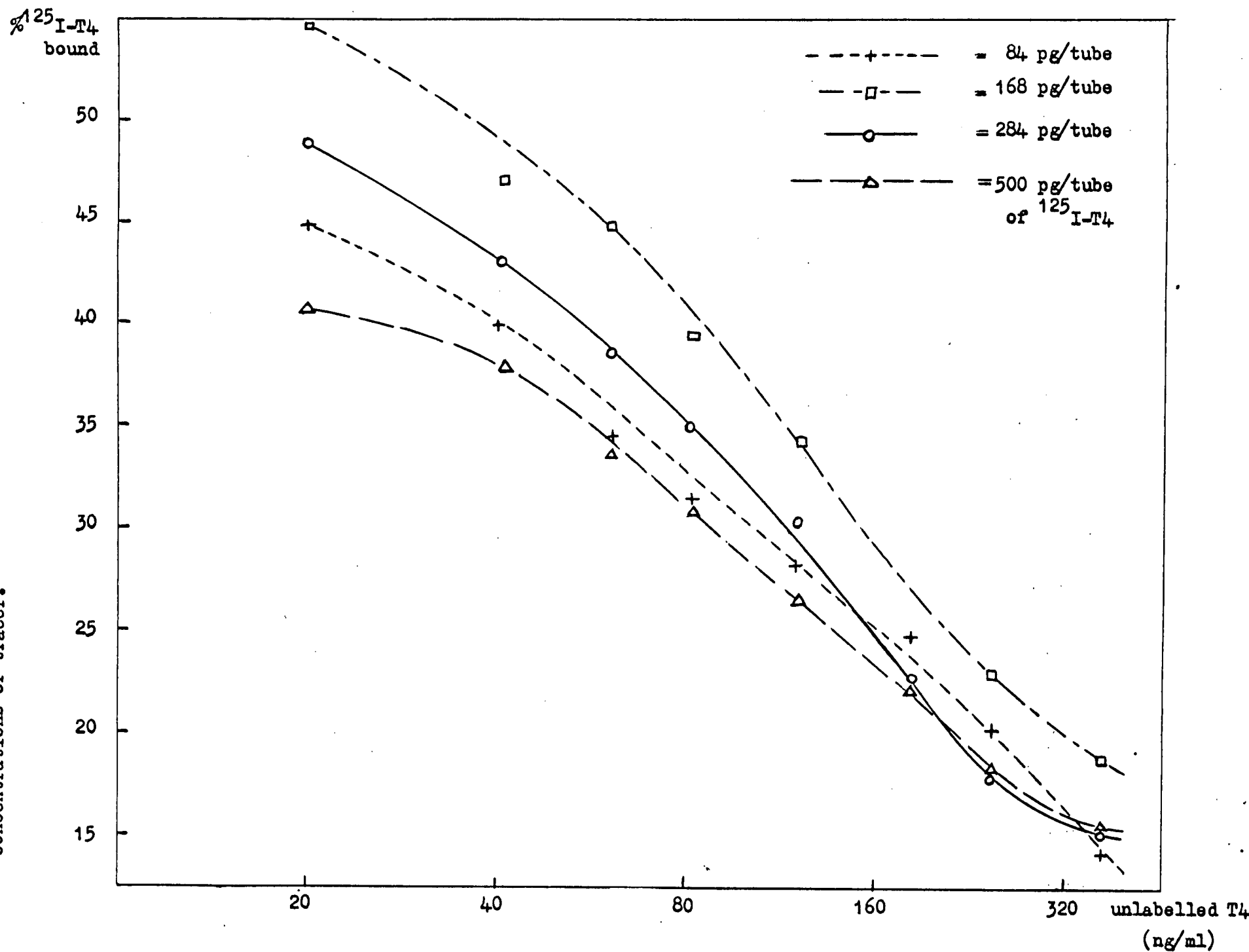


Figure 2.2 :

T4 assay optimization : graph showing standard curves at four different concentrations of tracer.



II.2 3,3',5-triiodothyronine solid phase radioimmunoassay.

II.2.1 Principle :

The same principles were applied to the T3 assay as to the T4 assay, including the use of insolubilized antibodies, incubation at high pH, addition of ANS and making up of the standards in T3-free serum. The method used was based on that of Seth, Toft and Irvine (1976).

II.2.2 Materials.

The T3 antiserum was raised in sheep and was kindly supplied by Dr. J. G. Ratcliffe, Stobhill General Hospital, Glasgow. The antiserum was coupled to cellulose by the same procedure as that used for T4 antiserum (see preceding section). The assay buffer, wash solution, sample cups and reaction tubes were also the same as for the T4 assay.

The T3 stock standard was prepared by dissolving 10 mg T3 free acid (Sigma) in propylene glycol/water (1:1 by volume) adjusted to pH 9.0 with NaOH. Working standards were prepared by serial dilutions of this stock standard in T3-free serum.

High specific activity ^{125}I - 3,3',5-triiodothyronine ($>1000 \mu\text{Ci}/\mu\text{g}$) was used in all assays. Initially this tracer ($1000 \mu\text{Ci}/\mu\text{g}$) was obtained from Cis-Sorin, Italy. Subsequently, tracer of activity $1090 \mu\text{Ci}/\mu\text{g}$ was used (NEN Chemicals, Winchester, Hants). Latterly, tracer with an activity of $1500 \mu\text{Ci}/\mu\text{g}$ was obtained from the Radiochemical Centre, Amersham. These changes were made as compounds became available which were progressively of higher activity, cheaper, and more readily obtainable.

The ANS/tracer working solution was prepared by adding 20 μCi $^{125}\text{I-T3}$ and 500 mg ANS to 400 mls assay buffer, giving a tracer concentration of 50 nCi/ml i.e. between 33 and 50 pg/ml, depending on the activity of the tracer. This solution was stored at 4°C .

II.2.3 Assay method.

Using the Analmatic sample preparation unit, 2 replicates of 50 μl of either standard or unknown plasma were picked up and dispensed into reaction tubes, each washed through by a 200 μl dispense of the ANS/tracer solution. At the same time, 500 μl of the solid-coupled antibody were dispensed from a stirred suspension in assay buffer to give a final antibody dilution of 10000- to 12000-fold in an incubation volume of 750 μl . Thus each incubation mixture for the standards contained 12.5 to 500 pg T3 in 50 μl T3-free serum (equivalent to 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 3.0, 6.0 and 10.0 ng/ml) while each tube for the unknowns contained 50 μl plasma. In addition all tubes contained 6.7 to 10 pg (= 10 nCi) $^{125}\text{I-T3}$ tracer (depending on the product used) and 250 μg ANS. The procedures for incubation, washing, centrifugation, decantation and counting were the same as in the T4 assay.

II.2.4 Assay optimization.

In most particulars the procedure followed was the same as for the T4 assay. However, the use of a high specific activity tracer is essential for a sensitive radioimmunoassay of T3. In the present assay, 10 pg or less of $^{125}\text{I-T3}$ were added per tube, depending on which tracer was used, which is less than the quantity of unlabelled hormone added in the bottom standard. The tracer could therefore be expected to provide sufficient

counts to determine the equilibrium ratio of free to antibody-bound T3 without significantly displacing that equilibrium. If, on the other hand, a low activity tracer had been used (say 50 $\mu\text{Ci}/\mu\text{g}$), it might have been necessary to add 200 pg $^{125}\text{I-T3}$ to obtain sufficient counts, to determine the free:bound ratio. Thus the quantity of labelled hormone per tube would exceed that of unlabelled hormone for more than half of the standard curve and the tracer would be contributing significantly to the observed equilibrium; this leads to lack of sensitivity in the presence of low levels of unlabelled hormone.

II.2.5 Sensitivity.

The hormone concentration corresponding to a level two deviations away from the zero standard was between 0.2 and 0.3 ng/ml in all assays, with a mean of 0.26 ng/ml.

II.2.6 Specificity.

Seth et al. (1976) found low cross-reactivity of this solid-coupled T3 antibody with T4, the latter having a reactivity of 0.0003 relative to the former. Thus a level of 300 ng/ml would add only 0.1 ng/ml to the apparent T3 concentration. The antibody showed quite high cross-reactivity with acetic and propionic acid analogues of triiodothyronine but the practical significance of this is uncertain.

II.3 Insulin double antibody radioimmunoassay.

II.3.1 Principle.

Insulin levels in plasma were determined by double antibody radioimmunoassay using a guinea-pig anti-insulin antiserum of high activity. A double equilibration system was used : standards (or unknown samples) and anti-insulin antiserum were incubated for 2-3 days at 4° C, after which the tracer was added and the system incubated for a further 2-3 days : finally, the antigen-antibody complex was precipitated using a donkey anti-guinea-pig gamma-globulin antiserum. After an overnight incubation, 1 ml assay buffer was added, the tubes were centrifuged and the supernatant decanted to waste. The precipitate was counted on an automatic gamma-counter with a paper tape output : the data from the tape were analysed as for T3 and T4 (see section on Statistical analysis of assays).

II.3.2 Materials.

The antiserum was a guinea-pig anti bovine insulin antiserum (Miles Laboratories, Slough, Bucks). The neat antiserum was diluted 1:100 in assay buffer and stored frozen in 1 ml aliquots.

The assay buffer was 0.15 mol/l phosphate saline buffer, pH 7.5, containing bovine serum albumin (5g/l) and sodium azide (100mg/l).

¹²⁵I-Insulin, specific activity 50 $\mu\text{Ci}/\mu\text{g}$, was obtained from the Radiochemical Centre, Amersham. The working solution of tracer was prepared by diluting 5 mls of stock solution to 100 mls in assay buffer. This dilution was performed immediately prior to use.

The insulin standard was six times recrystallized bovine insulin (Boots). The stock standard solution was prepared by dissolving 10 mg (240 mU) insulin in 5 mls dilute acetic acid (pH 3.0) which was then made up to 50 mls with assay buffer. Working standards for use in the assay were prepared by serial dilution of the stock solution in assay buffer.

The sample tubes used were polystyrene 3.0 ml cups (Sarstedt) and the reaction tubes were Luckhams LP4's.

II.3.3 Assay method.

Using the Analmatic sample preparation unit, 2 replicates of 200 μ l were picked up from the standard or unknown plasma sample and dispensed into reaction tubes, each washed through with 500 μ l assay buffer containing guinea-pig antiserum to bovine insulin (final dilution, 1:800,000). This mixture was incubated for 2-3 days at 4^o C. Each incubation mixture for the standards contained 0.0125 to 2.0 ng insulin (= 0.3 to 48.6 μ U) in 200 μ l assay buffer (equivalent to 0.0625, 0.125, 0.175, 0.25, 0.5, 0.75, 1.0, 2.5 and 10 ng/ml: or 1.5, 3.0, 4.2, 6.1, 12.15, 16.2, 24.3, 60.75 and 243 μ U/ml) while each tube for the unknowns contained 200 μ l plasma. Each standard was run in quadruplicate, each unknown in duplicate. After the first incubation 100 μ l tracer solution were added by automatic pipette (Compupet; General Diagnostics), this solution containing 100 pg (= 5 nCi) ¹²⁵I-insulin in 100 μ l. The incubation was then continued for 2-3 days at 4^o C. Pooled normal guinea-pig serum (100 μ l, final dilution 1:2200) was then added, followed by donkey antiserum to guinea-pig gamma-globulin (200 μ l, final dilution 1:150; Guildhay Antisera, University of Surrey, Guildford). All tubes were mixed in a vortex mixer after each reagent addition. After a further overnight incubation at 4^o C, 1 ml assay buffer

was added to each tube and they were centrifuged at 1200g for 30 min. The supernatant was decanted to waste, any remaining droplets being aspirated from the rim of the tube. The tubes were counted on an automatic gamma-counter for 100 seconds.

II.3.4 Assay optimization.

A series of experimental assays was carried out with the following aims :

- (i) to optimize the sample volume to ensure a reduction in the binding of labelled hormone by even small concentrations of unlabelled hormone.
- (ii) to ensure that the presence of 25% plasma in the incubation mixture did not per se affect the antigen-antibody reaction.
- (iii) to minimize non-specific binding by use of certain tubes, "clean" tracer and high protein concentration in the assay buffer.
- (iv) to optimize the concentrations of second antibody and normal guinea-pig serum to achieve full precipitation of bound tracer.
- (v) to optimize incubation times for full equilibration and best sensitivity.

Initially, tracer was prepared by iodination of bovine insulin (Boots) with Iodine-125 (Radiochemical Centre) in the laboratory. However, the combination of this tracer and the polystyrene tubes then used (Nunc UK, N-1075) gave very high non-specific binding, even when the tracer was run through a "clean-up" column immediately prior to use. By comparison, tracer bought direct from the Radiochemical Centre showed much lower non-specific binding : this was lowered further by the use of Luckham's LP4 polystyrene tubes. The system was then tested using 1, 2 or 5 g BSA

per litre in the assay buffer; the highest concentration gave least non-specific binding without affecting the antibody binding.

The guinea-pig anti-bovine insulin antiserum was titrated out so as to obtain about 35% binding of labelled antigen : this was found to be at a final dilution of 1:800,000.

Standards were prepared to cover the range from 90% to 10% of the binding of the zero-standard, using 50 μ l of standard solution per tube. However, 50 μ l of plasma did not give adequate reduction of binding to achieve a sensitive assay. Finally, 200 μ l of plasma were used and the standards were prepared four times more dilute so that the same range was covered using 200 μ l of standard per tube.

Combinations of normal guinea-pig serum at 1:50, 1:100, 1:200 and 1:400 and second antibody at 1:5, 1:10, 1:20, 1:30, 1:40 and 1:60 were added to zero-standard and blank tubes to optimize precipitation. The presence of plasma was found to have no significant effect on precipitation at this stage. EDTA was added throughout at the second antibody stage at a concentration of 2 mmol/litre in the incubation mixture to prevent interference in precipitation caused by complement in plasma.

Sensitivity was increased by using incubation times of at least 2 days before addition of tracer and a further 2 days before addition of 2nd antibody.

II.3.5 Sensitivity.

The least detectable concentration of insulin was taken as the point on the standard curve at which the confidence limits (± 2 S.D.) reached the mean level of binding for the zero-standards. The sensitivity of this assay was generally 0.07 ng/ml (= 1.7 μ U/ml) and corresponded to a percentage binding 87% of that of the zero-standard. Details of the standard curve and quality control will be found in the subsequent section II.4 on statistical analysis of assays.

II.3.6 Specificity.

There are apparently few proteins in plasma that show significant structural homology with insulin and might thus be expected to cross-react with the anti-bovine insulin antiserum. Generally speaking, non-specific effects tend to give rise to falsely high plasma values in radioimmunoassay : thus the sensitivity of the assay in situations where very low plasma values are expected is greater in the absence of non-specific effects, since these would represent apparent insulin above the underlying true concentration. Measured insulin values in plasma from animals after prolonged fasting were in some cases as low as 0.02ng/ml (0.5 μ U/ml) in the present assay. This suggests a satisfactory degree of specificity.

Growth hormone radioimmunoassay.

Growth hormone was measured in Experiment 3 by Dr. I. C. Hart, National Institute for Research in Dairying, Shinfield, Berks, by the method of Hart, Flux, Andrews and McNeilly (1975).

II.4 Statistical analysis of assays.

II.4.1 Radioimmunoassay program description.

All assay counting data were punched on to paper tape by the Wallac Autogamma Spectrometer and analysed using the ABRO data processing program package. The package consists of four programs.

The assay parameters, each identified by a directive, are input from a terminal. Parameters not present are assigned default values. Assay counts are input from paper tape and stored after preliminary checking. The output from program 1 comprises : errors in the parameter list or in assay counts, list of parameters including default values, list of assay counts and statistics for batch and overall standards.

In program 2 the standard counts are combined with the concentration level information from the input parameters. A calibration curve is calculated by applying the logit-log transformation and regression analysis to each batch of standards and to the overall standards. The control counts are treated in the same manner. The "edit" option allows the user to select or reject the tube counts for analysis thus overruling or supplementing the screening process. A Scatchard plot is available if requested. The output from program 2 includes the results of unweighted and weighted regressions and a plot of $\text{logit}(y) \text{ v } \log(x)$ for each batch of standards and overall standards (where y = response variable and x = dose) and for the controls.

Program 3 uses analysis of variance to examine the calibration curves calculated by program 2. Each batch standard curve and the overall curve

are tested for linearity, and the batches are tested for equality. The batch standard and control curves are combined and tested for linearity. The overall standard and control curves are tested for parallelism.

In program 4 potency estimates are calculated for each unknown sample. Upper and lower 95% confidence limits are calculated based on the pooled residual variance of the standard curve and the counts for the sample.

II.4.2 Data checking.

The output from program 1 allows easy detection of obvious outliers among the standards or of counts above zero-standard or below blank counts for which no interpolation would be possible. These can be rejected. By running zero-standards at points throughout the assay, a plot of their counts can be obtained in program 1 allowing easy visual assessment of drift. No significant drift occurred in the insulin assays by this assessment, and the equality of the two standard batches was accepted in all assays on an F-test. Although the plot of zero-standards showed tendencies towards drift in the T3 and T4 assays, the hypothesis of equality was rejected in one T4 assay only. Consequently this assay was divided into two sections and results for the unknowns in each section obtained from the batch standard curve in that section. Bias is unlikely to have occurred since the order of the unknown samples was randomized by a computer-generated sequence.

The F-test of homogeneity for the replicates of unknown samples generally had degrees of freedom $n_1 = 1$, $n_2 = 50$ in all assays. Although an F value of 5.0 is significant at the 5% level on these degrees of freedom, many values greater than this will occur when large numbers of independent

tests are performed (say 300 per assay). Thus the cut-off value for F was taken as 10.0 and unknown samples with F-values exceeding 10.0 for homogeneity were rejected and re-assayed.

II.4.3 Quality Control : Linearity, parallelism and slope of dose-response curves.

a) T₄ assays :

Linearity of the overall standard curve and parallelism of the standard and control curves were achieved in all assays (as judged by non-significance of F-tests for non-linearity, non-parallelism etc.). The slope of the standard curve (weighted regression of logit Y on $\log_e X$, as described) lay between -0.96 and -1.09 in all assays. Mean coefficients of variation for standard counts within assays ranged from about 2.0% for zero standards to about 7.0% for the top standard.

b) T₃ assays :

Linearity of the overall standard curve was achieved in all assays and parallelism of the standard and control curves in all assays except one, where replication of the control sample was poor. The slope of the standard curve lay between -0.91 and -1.01. Coefficients of variation for standard counts lay in the same range as the T₄ standards.

c) Insulin assays :

In all but one assay the F-test for linearity was rejected. Two contributing factors should be considered : first, the residual variance used as the denominator in the F-test was generally very low due to good replication of standards (coefficients of variation 2-3%); second, although the logit-log transformation empirically gives a good linear fit for many

sigmoid curves, there is no theoretical reason why it should do so, especially over a very wide concentration range (160-fold). Each standard was run in quadruplicate in each batch, as described. On inspection of potency estimates for unknown samples, most were found to lie below the mid-range of the standard curve. Subsequently, the assays were re-run using the edit facility to remove some of the higher standards so that they received less weighting in the plotting of the curve : for example, all standards less than 1.0 ng/ml might be represented by eight tubes while higher standards were represented by four tubes only. Mean slopes of the standard curves before editing lay between -0.82 and -0.90; after editing the range was -0.89 to -0.97. In each case the slope after editing was nearer the slope of the overall control curve.

II.4.4 Quality Control : Comparison with independent assay methods.

For each hormone assay system, serum control samples for which potency estimates had been obtained in independent laboratories were run in each of the first two assays. Control samples for T₄ and T₃ were supplied by Dr. Seth and those for insulin were supplied by Dr. Hart. The corresponding comparisons among the results obtained are shown in Tables 2.1, 2.2 and 2.3. In the remainder of the assays quality control was estimated as described in the next section.

II.4.5 Quality Control : Between and within assay variation.

For each hormone assay system between and within assay variation was estimated as follows : with each batch of standards a pooled bovine plasma sample was run in duplicate at concentrations of 50% and 100%. Thus four potency estimates were obtained for this sample in each assay, each derived from a pair of tubes but on different sections of the standard

curve. All these potency estimates for all assays were tabulated and a one-way analysis of variance was performed.

For any pair of replicates of an unknown sample, a mean potency estimate can be obtained with expected variance σ_e^2 , the within-assay or error variance. For any pair of replicates in separate assays, the potency estimate will have the same expected mean but an expected variance of $\sigma_e^2 + \sigma_A^2$, where σ_A^2 is the between-assay component. Estimates of σ_e^2 and σ_A^2 were obtained by the analysis described which gave results of the form :

<u>Source of variation</u>	<u>Expectation of mean square</u>
Assay	$\sigma_e^2 + 4\sigma_A^2$
Residual	σ_e^2

The coefficient of variation % between assays was taken as : $\frac{\sqrt{\sigma_e^2 + \sigma_A^2}}{\mu} \times 100$,

where μ = mean potency estimate. The coefficient of variation % within assays was taken as $\frac{\sqrt{\sigma_e^2}}{\mu} \times 100$.

For the insulin assay, within assay variation was 8.6% and between assay variation was 10.3%.

For the T4 assay, within assay variation was 7.8% and between assay variation was 12.8%.

For the T3 assay, within assay variation was 6.9% and between assay variation was 8.9%.

Table 2.1 :

Comparison of T₄ values for control samples with an independent assay method.

	Dr. J. Seth	Present assay	
		Assay 1	Assay 2
Low pool	47-72 nmol.l ⁻¹	40.5 ng/ml (= 52.2 nmol/l)	45.9 ng/ml (= 59.1 nmol/l)
Medium pool	102-133 nmol.l ⁻¹	93.7 ng/ml (= 120.7 nmol/l)	100.1 ng/ml (= 129.0 nmol/l)
High pool	208-253 nmol.l ⁻¹	167.0 ng/ml (= 215.2 nmol/l)	

Table 2.2 :

Comparison of T₃ values for control samples with an independent assay method.

	Dr. J. Seth	Present Assay	
		Assay 1	Assay 2
High pool	1.12 nmol.l ⁻¹ = 0.73 ng/ml	0.99 ng/ml	1.07 ng/ml
Medium pool	2.08 nmol.l ⁻¹ = 1.35 ng/ml	1.40 ng/ml	1.42 ng/ml
Low pool	3.61 nmol.l ⁻¹ = 2.35 ng/ml	2.17 ng/ml	2.21 ng/ml

Table 2.3 :

Comparison of insulin values for control samples with an independent assay method.

	Dr. Hart	Present assay	
		Assay 1	Assay 2
Control A	11.4 uU/ml	12.26 uU/ml	13.04
Control B	10.5 uU/ml	12.21	11.38

CHAPTER III : HORMONE MEASUREMENTS IN FRIESIAN BULLS UNDERGOING PROGENY

TESTS.

III.1 Introduction.

In the U.K. young dairy bulls undergoing progeny tests for milk production are 'laid off' for 4-5 years between the time of semen collection and the analysis of their daughters' lactation records. It was decided to investigate hormone levels in a group of these bulls to determine whether aspects of their own energy metabolism might relate to their daughters' future achievements in milk production. The hormones chosen for study were insulin, thyroxine (T₄) and triiodothyronine (T₃) for the reasons given in chapter I. The advantages of using AI bulls as experimental animals are :

- 1) their breeding value can be estimated with considerable accuracy
- 2) the environmental covariance between hormone levels and milk yield can be neglected
- 3) the genetic variation for milk production among this group of bulls corresponds to the sort of range of variation within which a physiological selection criterion might be useful.

Because of the current predominance of British Friesian bulls used for insemination of dairy cattle in the U.K., it was decided to study bulls in this breed only.

III.2 Materials and Methods.

III.2.1 Bulls.

The animals sampled were 178 Friesian bulls between 2 and 7 years old : 149 at the Milk Marketing Board (M.M.B.) farm at Whenby Lodge, Brandsby, North Yorkshire : 14 at the M.M.B. Cattle Breeding Centre, Beccles, Suffolk; and 15 at the M.M.B. Cattle Breeding Centre, Calthwaite, Penrith, Cumbria. All were housed indoors.

III.2.2 Experimental Methods.

10 ml blood samples were taken from a jugular vein into heparinized tubes using the Vacutainer system (Becton and Dickinson). After 20 minutes' centrifugation, plasma was pipetted off and stored frozen. Each bull was sampled twice with a 3-day interval between the samples and the sampling sequence was reversed on the second occasion. The time of day and the time elapsed since feeding were recorded for each sample.

Hormone concentrations were measured as described (see chapter II, Assay methods). All the plasma samples were run in a single assay for each hormone except for a small number (about 5%) which had to be re-assayed. Groups of twenty samples were randomized in sequence for the assay.

III.2.3 Statistical Methods.

Progeny test results were obtained in the form of improved contemporary comparisons (ICC's). The ICC is derived from comparison of the first lactation records of one sire's daughters with those of other sires' daughters in the same breed, herd and year (M.M.B., 1974). By June 1978,

ICC values were available for bulls born in 1971, 1972 and some of those born in 1973. Of the 178 bulls sampled, 100 had ICC's for milk yield with 'effective daughter' weightings greater than 10, and 81 of these had ICC's with weightings greater than 20. The ICC values ranged from + 542 kg milk to - 287 kg, with a mean of + 101 kg, which corresponds closely to the reported mean ICC of all M.M.B. bulls born in 1971 which was + 100 kg (M.M.B., 1978).

Hormone levels were subjected to least-squares analysis of variance using Harvey's program (1960). The following effects were examined :

- 1) Sires of bulls (73 sires)
- 2) Station (1, 2 or 3)
- 3) ICC (as a continuous variable, range - 287 to + 542)
- 4) Age (as a continuous variable, range 2 to 7 years)
- 5) Bulls
- 6) Time of day (as a continuous variable, range 0900 - 1600 hours)
- 7) Time after feeding (as a continuous variable, range 40 - 180 minutes).

Sire effects did not account for significant variation in any of the hormones studied, F-values for the tests of sire effects against bull effects being less than 1 in each case. Effect of sire was thereafter dropped from the model and sires were considered as being independent.

Because of possible confounding between effects, for instance between age and ICC, it was decided to fit regressions for factors 3), 4), 6) and 7) in a single analysis rather than correcting for 4), 6) and 7) before fitting ICC. In order to do this, it was necessary to fit a

dummy variable, 'Yes-ICC', to separate bulls which did have ICC's from those which did not. All bulls without ICC's were assigned a notional ICC value (which does not affect the regression coefficient : this was checked by running a further analysis including only data from bulls which did have ICC's).

Preliminary analysis had shown that none of the quadratic or higher order regressions were significant, so that only linear terms were included in the final analysis of variance table which was constructed from the following two analyses :

<u>Analysis 1</u>		<u>Analysis 2</u>	
<u>Effect</u>	<u>d.f.</u>	<u>Effect</u>	<u>d.f.</u>
Mean	1		
"Yes-ICC"	1		
Station	2		
Age	1		
ICC	1	Bulls	175
Time of day	1	Time of day	1
Time after feeding	1	Time after feeding	1
Residual	334	Residual	165

The between-bulls mean square was obtained by subtracting the residual sum of squares obtained in analysis 1 from that obtained in analysis 2 and dividing by the corresponding degrees of freedom (159). The between-bulls component of variance was estimated by equating the bulls mean square to its expectation and a repeatability was calculated for each hormone as $\frac{\sigma_B^2}{\sigma_B^2 + \sigma_W^2}$ where σ_W^2 is the residual mean square.

Analysis 1 was repeated twice, once using only the ICC values with weightings greater than 20 to estimate the ICC regression, and a second time using only ICC's less than - 100 kg or greater than + 200 kg milk, also with weightings over 20. In both cases the estimated regression coefficients were similar to those obtained in the original analysis using all the ICC values.

III.3 Results.

Overall mean concentrations were 0.85 ng/ml for insulin, 1.21 ng/ml for T3 and 81.3 ng/ml for T4. Analysis of variance tables for each hormone are given in table 3.1. It can be seen that all of the environmental effects were significant for at least one of the hormones studied : station effects and time of day effects for insulin and T4, age and time after feeding for T3. For none of the hormones studied did ICC account for significant variation, although bull effects were significant for T3 and T4. Estimates of the regression coefficients obtained are given in table 3.2. Again it can be seen that for ICC none differed significantly from zero.



Table 3.1 :

Hormone levels in Friesian bulls : analysis of variance for dependent variables.

Source	d.f.	T3		T4		Insulin	
		Mean square	F	Mean square	F	Mean square	F
Station	2	0.554	1.67	2029.7	4.66*	36.0464	86.82***
ICC (regression)	1	0.247	0.74	718.1	1.65	0.0014	0.00
Age (regression)	1	6.537	19.69***	208.0	0.48	0.0164	0.04
Between bulls	159	0.332	2.17***	435.2	1.40*	0.4152	1.22
Time of Day (regression)	1	0.064	0.42	2293.8	7.38**	1.8250	5.35*
Time after feed (regression)	1	1.782	11.62***	710.2	2.29	0.2576	0.76
Residual	165	0.153		310.6		0.3411	

Table 3.2 :

Hormone levels in Friesian bulls : least-squares fitted values for each effect.

	T3	T4	Insulin
Overall mean	1.213	81.3	0.845
Station : Whenby	1.079	80.8	0.793
Penrith	1.305	94.2	2.667
Beccles	1.330	72.8	0.145
ICC regression (ng/ml/100 kg milk)	- 0.0207 ± 0.020	- 1.114 ± 0.789	- 0.0016 ± 0.025
Age regression (ng/ml/year)	- 0.127 ± 0.024	+ 0.714 ± 0.940	- 0.006 ± 0.029
Time of day regression (ng/ml/hour)	+ 0.0094 ± 0.015	+ 1.789 ± 0.658	- 0.0505 ± 0.022
Time after feed regression (ng/ml/min.)	+ 0.0038 ± 0.0011	- 0.077 ± 0.051	+ 0.0015 ± 0.0017
Repeatability ($\sigma_B^2 / \sigma_B^2 + \sigma_W^2$)	0.368	0.167	0.098

III.4 Discussion.

The main aim of the present experiment was to identify possible relationships between hormone levels and ICC. However, none of the regression coefficients for thyroxine (T₄), triiodothyronine (T₃) or insulin on ICC were significant. Because ICC values are regressed for error, the regression coefficients obtained are unbiased estimates of those that would have been obtained if ICC values had been known without error (Taylor et al., 1978). This was borne out to some extent by the fact that coefficients obtained by using only data from bulls with high weightings were little affected.

Joakimsen et al. (1971, 1975) found positive correlations between the thyroxine degradation rate (TDR) of dairy bulls and their daughters' lactational performance. Joakimsen decided that plasma levels of T₄ alone did not adequately reflect thyroid activity and instead estimated degradation rate by injecting radioactive thyroxine and measuring its disappearance rate from blood over two days. TDR is calculated as the product of 1) plasma T₄ level 2) fractional disappearance rate of labelled T₄ and 3) distribution volume, derived from the reciprocal of the observed dilution immediately after injection.

In their first experiment, Joakimsen and colleagues estimated TDR in mature bulls of four breeds and found positive correlations ranging from 0.14 to 0.47 between bulls' TDR and their daughters' lactation yield. Although the correlations reached significance in only one breed, the pooled correlation across breeds was highly significant ($r = 0.30$, $n = 82$). Correlations between plasma T₄ levels and daughters' milk yield were not given.

In their second experiment, they measured TDR and also plasma triiodothyronine (T3) levels in two groups of young bulls between 4 and 16 months old (see chapter I, table 1.1). They found no fixed relationship between T3 and daughters' lactation yield ($r = 0.03$ and $- 0.09$ in the two groups). Since the F-test for the equivalent regressions would be $\frac{r^2(n - 2)}{1 - r^2}$ with $r = 80$ and $n = 4$ for the two groups, it can be seen that

the regressions would be non-significant as in the present study.

Correlations between TDR and daughters' performance were again small and positive but non-significant.

The two main advantages of measuring TDR are, first, that the measurement is made in a 'dynamic' situation rather than measuring basal plasma T4 levels in a 'static' situation; second, in functional terms, it does appear to show a consistent positive correlation with daughters' milk yield. Correspondingly, there are several drawbacks : first, it is not always feasible or desirable to inject radioisotopes into breeding bulls. Second, the thyroxine distribution volume is estimated by plotting the decay curve on a log scale and extrapolating a straight line back to time zero; but this assumes that the body acts as a single rapidly equilibrating compartment for T4, an assumption which has been questioned (Oppenheimer et al., 1975). Finally, in physiological terms it is not only the rate of T4 degradation which is important, but the manner of degradation : for deiodination of T4 at the 5' position leads to the production of T3, several times more potent than T4, whereas deiodination at the 5 position produces reverse T3, a hormone with almost no calorogenic effect (Chopra et al., 1975). Further experiments are being undertaken in Norway to try to clarify the relationship among TDR, T4, T3, milk production and feed efficiency (N. Standal, personal communication).

Repeatabilities, estimated from intraclass correlations between successive measurements, were lower in the present study than in the Norwegian work : values of 0.37 and 0.17 were obtained for T3 and T4 respectively, compared with 0.48 for T3 (Joakimsen, 1975) and about 0.6 for T4 (calculated from Table 2, Joakimsen et al., 1971). The repeatability estimate for insulin was even lower (0.10) and environmental effects were large, particularly that of station : bulls from station 3 had mean insulin levels 3-fold higher than those at stations 1 and 2. This difference probably arises from the quality or quantity of feed given, but the time of feeding had no significant effect. For T3 and T4, other environmental effects were found to be significant : the negative relationship between T3 and age is consistent with results from adult men (Chopra et al., 1975).

Tucker et al. (1974) measured serum prolactin and growth hormone in Holstein bulls but found neither hormone to be significantly related to breeding value for milk yield. They concluded that "basal estimates of these hormones will have little usefulness in selection processes for dairy cattle until sampling variation is more completely defined or controlled". The present results would support these conclusions for insulin, T4 and T3.

Stark, Rowlands, Manston and McClintock (1978) analysed samples from a comparable group of Friesian bulls to those in the present study and found significant correlations of ICC with blood urea ($r = 0.24$) and inorganic phosphate ($r = - 0.24$), but they did not discuss their possible physiological significance. Their results, together with the other results discussed in this section, may suggest that greater emphasis should be placed on

using intensive rather than extensive (survey-type) sampling regimes. This would allow a) reduction of environmental effects b) manipulation of treatment where necessary, e.g. application of suitable stimuli, in order to study the dynamic aspects of metabolism c) deeper study of basic physiological processes of energy metabolism, e.g. protein synthesis and breakdown, which might relate both to TDR and to urea levels.

CHAPTER IV HORMONE MEASUREMENTS IN FRIESIAN AND JERSEY CALVES OF HIGH
OR LOW GENETIC MERIT FOR MILK PRODUCTION.

IV.1 Introduction.

In 1968 an experiment was set up at ABRO to investigate the efficiency of production in Friesian and Jersey cattle (Monteiro 1972, 1974, 1975). The experiment was designed for efficient estimation of differences between the progeny of two groups of bulls, one group having high and the other group low breeding values for milk production. It was decided to investigate hormone levels in these progeny to determine whether aspects of their physiology might reflect their known differences in genetic merit for milk production.

IV.2 Materials and Methods.

Friesian and Jersey cows were allocated at random either to high contemporary comparison (CC) bulls for their first pregnancy and to low CC bulls for their second pregnancy or vice versa. Within each breed, two groups of four proven bulls were chosen from those with the highest and lowest CC's available in 1969. The mean values for the 4 groups in 1971 CC values were : Friesian "high", + 435 kg milk; Friesian "low", - 492 kg; Jersey "high", + 232 kg; and Jersey "low", - 134 kg. The animals in the present experiment were the pure bred progeny of these matings. From weaning, all animals were fed ad libitum on the ARC AA6 complete diet and housed indoors. At ages of 12, 24, 36, 48, 60 and 72 weeks 10 ml blood samples were taken from the jugular vein into heparinized tubes using the Vacutainer system. After centrifugation, plasma was removed and stored frozen. All samples were taken between 9.00 and 10.30 a.m.

Because of the continuous nature of experiment in which these animals were involved, the calves studied covered the whole range from 12 to 72 weeks both at the beginning and the end of the present investigation. Thus although some calves were represented by six samples, many were represented by less. Overall, 204 samples were taken from 68 calves over a period of 2 years.

Insulin, T₄ and T₃ levels in plasma were measured as described (chapter II, Assay Methods). All the plasma samples were run in a single assay for each hormone except for about 5% which had to be re-assayed.

IV.2.2 Statistical methods.

A series of three least-squares analyses was performed using Russell's (1979) Compreg program. The four dependent variates were T4 level, T3 level, insulin level and body weight. The independent variates (effects) examined were 1) mean 2) breed - Friesian or Jersey 3) CC group of sire - high or low 4) breed x CC group interaction 5) sire effects within breed and within CC group 6) sex 7) individual effects within sires 8) age - 12, 24, 36, 48, 60, or 72 weeks 9) year-season of sample - as eight 3-month periods. It was not possible to fit dams because only a small number of dams was represented by more than one progeny in the two-year period of the present experiment. Numbers in subclasses for each effect are shown in table 4.1.

In the first analysis, individuals were absorbed and the factors age and year-season were fitted. The residual mean square was taken as the variance within individuals, and the effects due to age and year-season were tested against it.

In the second analysis, the factors fitted were the mean, sires, sex, age and year-season. The residual sum-of-squares (SS) was adjusted by subtracting the residual SS obtained in the first analysis to obtain a residual SS for estimation of the variance between individuals within sires. The effect due to sex was tested against this variance.

In the third analysis, the factors fitted were the mean, breed, CC group, breed x CC group interaction, sex, age and year-season. The residual SS was adjusted by subtracting the residual SS obtained in the second

analysis to give a residual SS from which the between-sire variance was estimated. The effects due to breed, CC group and the interaction of the two were tested against this variance.

In addition the variance between sires was tested against the variance between individuals, which in turn was tested against the variance within individuals. These procedures were followed for all four dependent variates.

Fitted values of each dependent variate were obtained for all levels of each factor. Residual correlations among dependent variates were obtained after adjusting for all factors.

Sire effects could not be regarded as random for the purpose of estimating variance components; the repeatability for each hormone was therefore estimated within sires by the method of Becker (1967).

Table 4.1 :

Numbers of measurements in subclasses for each independent variate.

Mean	204			
Breed	Friesian : 86		Jersey : 118	
CC group	High : 48	Low : 38	High : 45	Low : 73
Sire	16,12,11,9	13,8,10,7	16,12,10,7	23,23,16,11
Sex	Male : 102		Female : 102	
Age	12 weeks : 47 48 weeks : 30	24 weeks : 48 60 weeks : 23	36 weeks : 34 72 weeks : 22	
Year-season of sample	Jan-Mar	Apr-Jun	July-Sept	Oct-Dec
	1976	-	18	26
	1977	26	27	31
	1978	24	-	-

IV.3 Results.

Least-squares fitted values of the dependent variates for each effect are shown in tables 4.2 and 4.3. Age had a significant effect on insulin and T₃; graphs of hormone levels and body weight against age are shown in figures 4.1 to 4.4. Year-season had a significant effect on insulin and T₄. For the three hormones, none of the other effects were significant and the between individuals variance was only significant for T₄ (see table 4.4). Components of variance due to individuals were small; the repeatability estimates for T₄, T₃ and insulin were 0.17, 0.04 and 0.10 respectively (table 4.6). Levels of T₄ and T₃ were correlated ($r = 0.24$, $P < 0.01$; table 4.5) while all three hormones showed small positive correlations with body weight, but only for insulin was the correlation statistically significant (table 4.5).

Table 4.2 :

Fitted values of each dependent variate for genetic effects.

	T4	T3	Insulin	Body weight
Overall mean	44.7 ng/ml	1.46 ng/ml	0.343 ng/ml	171.5 kg
Friesian 'high' (FH)	44.9	1.48	0.390	239.2
Friesian 'low' (FL)	38.3	1.46	0.366	247.8
Jersey 'High' (JH)	45.7	1.56	0.328	173.8
Jersey 'low' (JL)	49.9	1.54	0.348	173.8
Sires : FH1	50.7	1.40	0.392	223.9
FH2	43.5	1.41	0.422	248.8
FH3	44.9	1.53	0.372	241.4
FH4	37.6	1.70	0.385	250.0
FL1	47.3	1.65	0.431	244.5
FL2	36.7	1.36	0.240	255.2
FL3	37.0	1.30	0.396	264.6
FL4	24.9	1.59	0.350	230.1
JH1	54.0	1.88	0.368	180.4
JH2	43.3	1.51	0.358	174.5
JH3	25.6	1.36	0.239	170.1
JH4	59.2	1.41	0.311	163.1
JL1	51.4	1.53	0.247	168.6
JL2	43.8	1.51	0.346	178.0
JL3	50.9	1.40	0.380	170.1
JL4	58.6	1.83	0.550	178.6
Male	45.9	1.55	0.382	217.2
Female	42.7	1.49	0.342	200.5

Table 4.3 :

Fitted values of each dependent variate for environmental effects.

	T4	T3	Insulin	Body weight
Overall mean	44.7 ng/ml	1.46 ng/ml	0.343 ng/ml	171.5 kg
Age : 12 weeks	26.4	0.98	0.398	57.6
24 weeks	37.5	1.34	0.163	109.5
36 weeks	42.0	1.78	0.249	174.8
48 weeks	55.8	1.74	0.327	246.3
60 weeks	55.7	1.73	0.476	303.1
72 weeks	50.0	1.55	0.587	360.9
Year-season :				
Apr-Jun '76	22.4	1.78	0.151	206.7
Jul-Sept '76	24.4	1.44	0.182	203.0
Oct-Dec '76	22.8	1.38	0.377	225.5
Jan-Mar '77	26.4	1.20	0.177	216.7
Apr-Jun '77	24.3	1.39	0.153	210.7
Jul-Sept '77	57.0	1.55	0.489	208.4
Oct-Dec '77	81.1	1.69	0.661	203.7
Jan-Mar '78	96.1	1.76	0.704	195.1

Table 4.4 : Analysis of variance for dependent variates.

Source	d.f.	T ₄		T ₃		Insulin		Body wt.	
		Mean square	F	Mean square	F	Mean square	F	Mean square	F
Breed	1	1860.1	2.00	0.360	0.94	0.0708	0.85	227082	192.93***
CC group	1	67.2	0.07	0.111	0.03	0.0005	0.01	1313	1.11
Breed x CC group	1	1404.2	1.51	0.003	0.00	0.0222	0.27	923	0.78
Sires	12	927.6	1.36	0.385	1.03	0.0833	0.64	1177	0.86
Sex	1	338.0	0.49	0.150	0.40	0.0484	0.37	9359	6.82*
Individuals	51	681.6	1.56*	0.373	1.11	0.1300	1.32	1372	3.81***
Age	5	937.9	2.15	1.572	4.70***	0.6191	6.27***	8542	23.74***
Year-season	7	4057.6	9.31***	0.289	0.86	0.5072	5.14***	781	2.17*
Residual	124	435.9		0.335		0.0987		360	

Table 4.5

Residual correlations among dependent variates after adjusting for sire, sex, age and year-season (averaged over all records).

	Body wt.	Insulin	T4
Insulin	0.149*		
T4	0.135	0.003	
T3	0.146	- 0.099	0.244**
* = P<0.05		** = P<0.01	D.f. = 174

Table 4.6 : Variance components and repeatability estimates.

Source of variation	Expectation of mean square
Between individuals	$\sigma_W^2 + K\sigma_B^2$
Residual	σ_W^2

	T4	T3	Insulin
σ_W^2	435.9	0.335	0.0987
σ_B^2	87.1	0.013	0.0110
Repeatability	0.167	0.039	0.101

The K-value was calculated as 2.82 by the method of Becker (1967)

Figure 4.1 : Graph of mean body weight versus age in dairy calves.

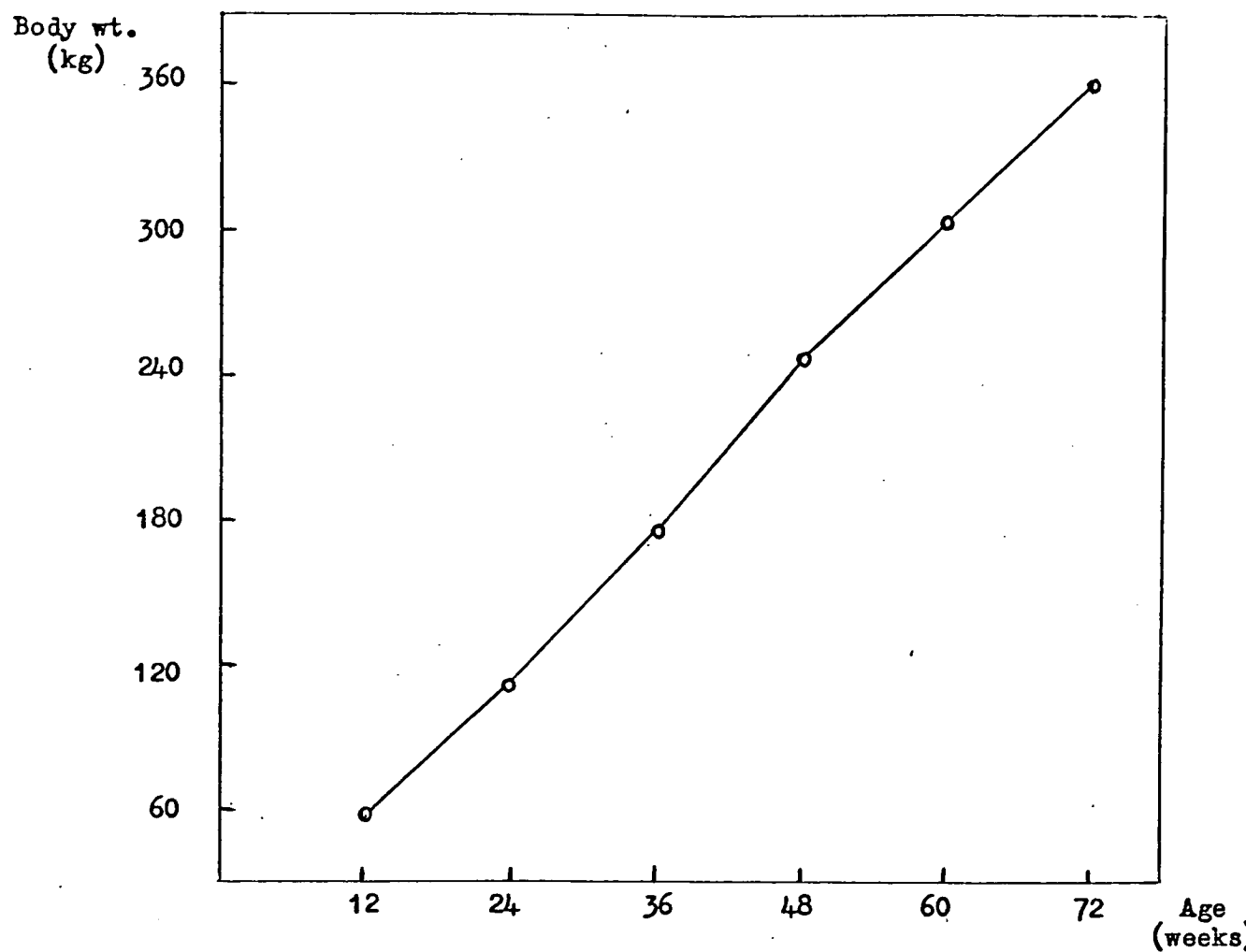


Figure 4.2 : Graph of mean insulin levels versus age in dairy calves.

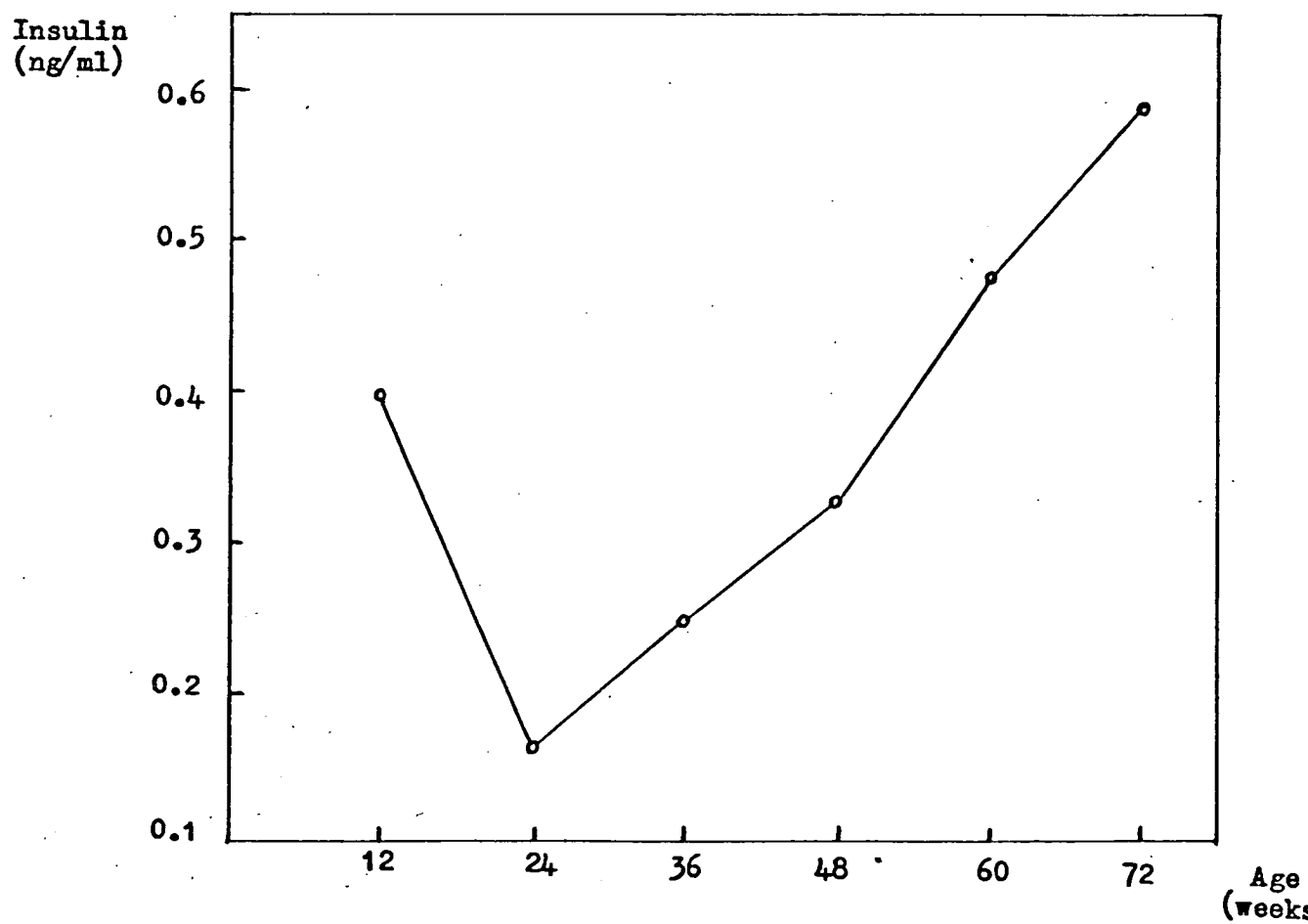


Figure 4.3 : Graph of mean T3 level versus age in dairy calves.

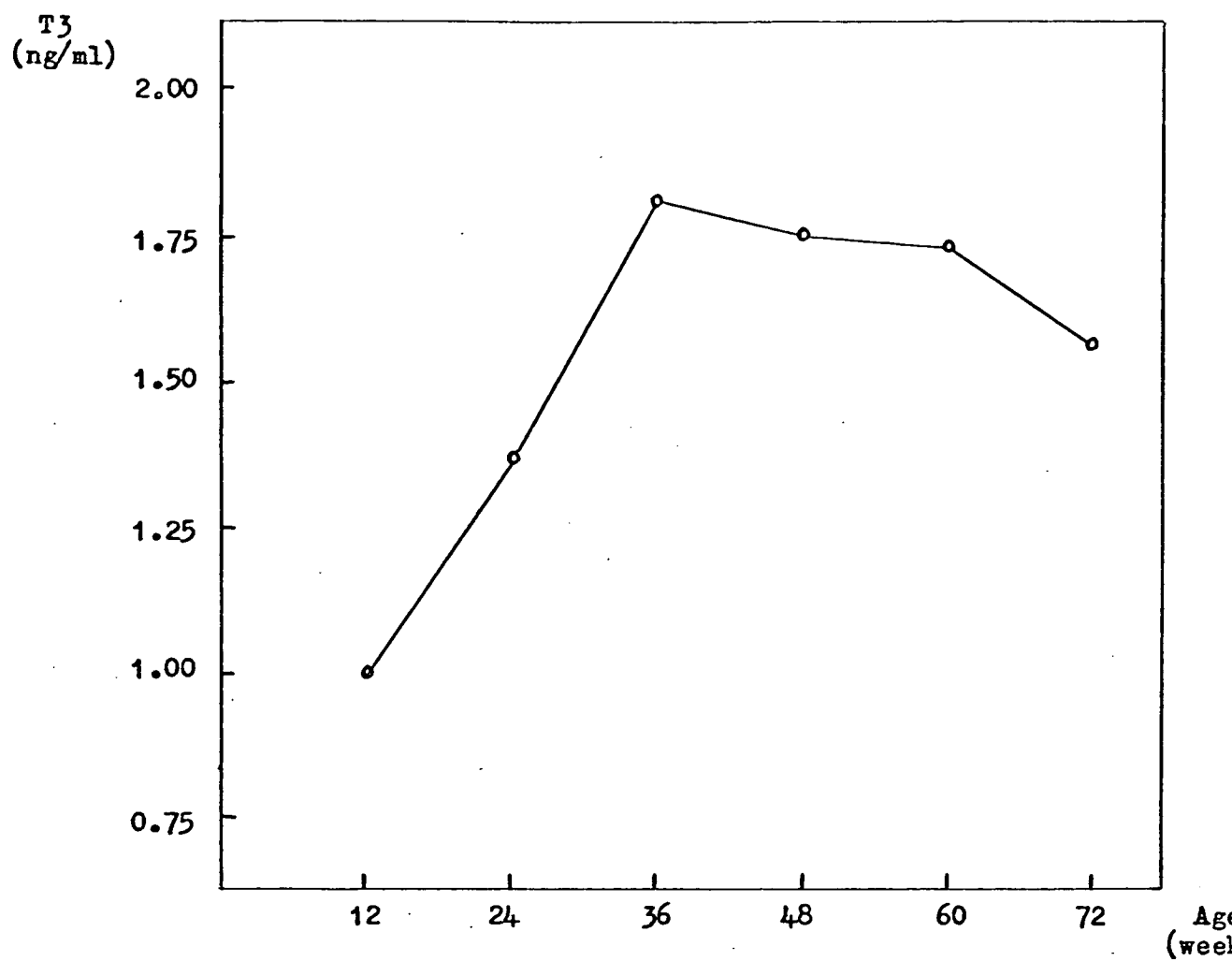
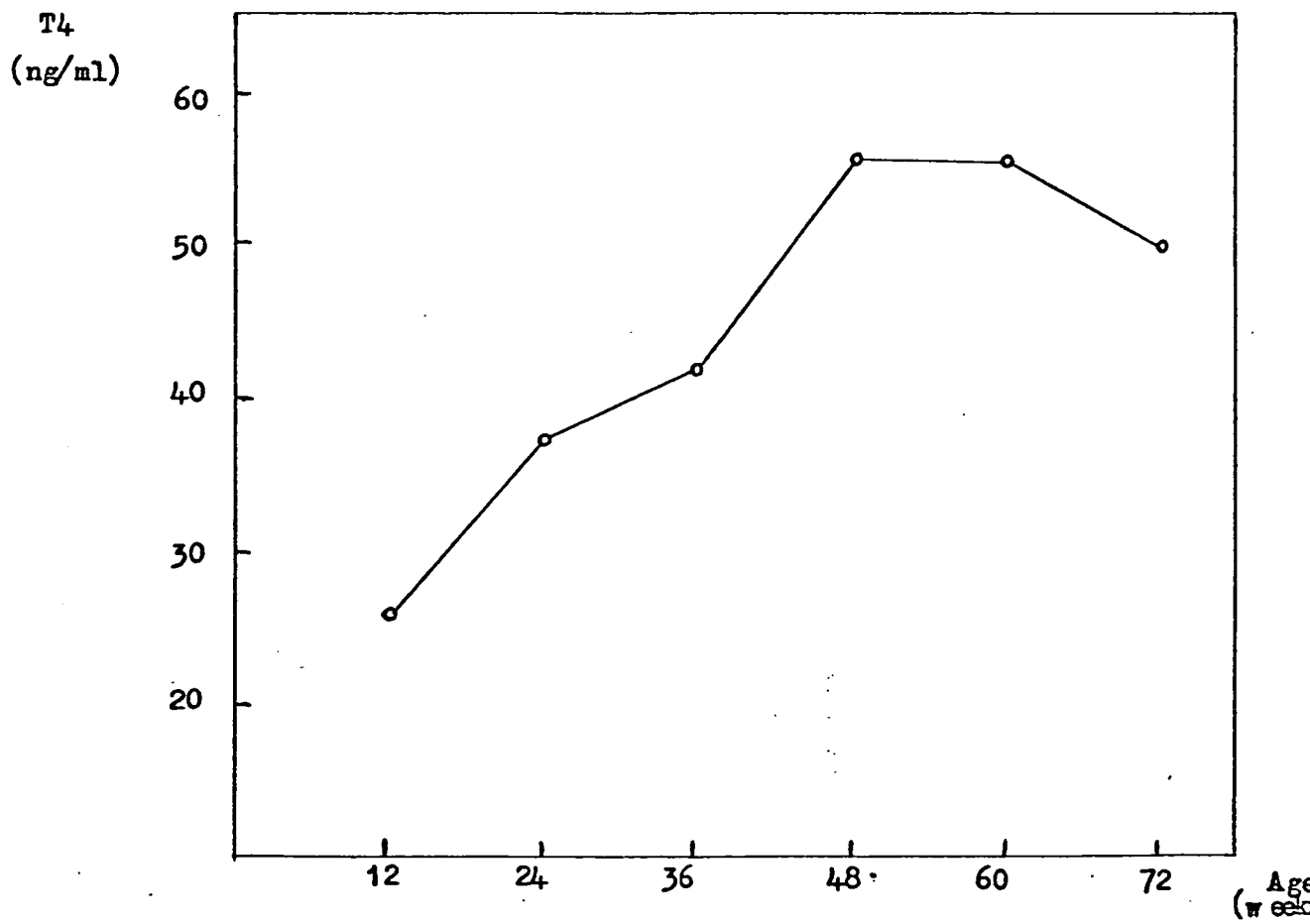


Figure 4.4 : Graph of mean T4 level versus age in dairy calves.



IV.4 Discussion.

There were no significant differences between breeds, between CC groups or sires within breeds and groups for any of the three hormones. Irvin and Trenkle (1971) found no effect of breed or sex on insulin and protein-bound iodine (a measurement approximating to T₄ level) in Angus, Hereford, Shorthorn and crossbred calves. In the present study all the genetic factors had small effects while the environmental factors, age and year-season, had fairly large effects. Similarly, variance due to sires and to individuals tended to be small relative to the residual variance for all hormones, and the repeatability estimates were low, especially for insulin and T₃. Joakimsen (1975) measured T₄ in 98 dairy bulls on four A.I. stations and his results indicate a repeatability for T₄ of about 0.60 (from Joakimsen et al., 1971; table 2). Joakimsen (1975) also measured T₃ in 94 young dairy bulls on four occasions between 4 and 16 months of age and estimated the repeatability for T₃ as 0.48. Bard (1973) measured T₄ in 159 beef bulls and, using a half-sib analysis, estimated the heritability of T₄ as 0.31. Measurement of insulin levels in 97 Angus bull calves at 18 and 30 weeks of age gave a correlation of 0.37 between insulin levels at the two ages (Erb, Stewart, Martin and Malven, 1979). The reason for the lower figures obtained for all three hormones in the present study is not clear.

Both the thyroid hormones rose markedly up to the age of 48 weeks but then reached a plateau and fell slightly at later ages. Insulin levels, although higher at 12 weeks than 24 weeks, rose consistently thereafter. A possible explanation for the higher levels of insulin at 12 weeks is

that rumination may not have been fully established. If this was so, a greater proportion of dietary energy may have been absorbed as glucose, tending to give higher levels of insulin. The tendency for the levels of all three hormones to rise with age may be due to body weight or food intake. The residual correlations between hormone levels and body weight were positive in all three cases, even after adjusting for age. Positive correlations have been reported in bulls between insulin and daily gain ($r = 0.35$; Erb *et al.*, 1979) and in pigs between insulin and fatness as measured by the ratio of subcutaneous fat wt. : longissimus dorsi muscle wt. ($r = 0.98$; Gregory *et al.*, 1976). Insulin levels in Holstein calves have been shown to rise considerably in the 13 weeks post-weaning (McGuffey, Thomas and Convey, 1977). Levels of T4 and T3 in calves have been shown to rise consistently between 2 and 22 weeks of age (Kahl *et al.*, 1977) and positive correlations has been reported between T4 and body weight with values of 0.20 and 0.34 for two different breeds (Bard, 1973).

Year-season had a significant but not consistent effect on T4, but insulin levels tended to be higher during the last 6 months of the 2-year experimental period. The reason for this is not clear but is unlikely to be due to degradation of insulin during prolonged storage at -20°C since Trenkle (1972) found insulin to be stable to at least one year's storage and to multiple freezing and thawing.

In the present study, therefore, genetic effects on hormone levels were low in comparison to environmental effects, particularly age. The low values obtained for repeatability estimates are perhaps due to the range

of ages at sampling, with the animals being immature and growing throughout the study. These results appear to indicate that despite attempts to standardize environmental conditions where possible, single samples over a range of ages do not have a sufficiently high repeatability to characterize a calf adequately in terms of its genetic merit for milk production. The relationship between the three hormones studied and age and body weight seems interesting and perhaps merits further study. However, in order for the physiological study of young calves to have any genetic application, it would seem necessary, at least for these three hormones, to take a larger number of blood samples per animal over a shorter time period so as to achieve a higher repeatability of measurement.

CHAPTER V : HORMONE STUDIES ON DAIRY AND CROSSBRED CALVES BEFORE AND
AFTER WEANING.

V.1 Introduction.

Although the differences in production characteristics among different breeds of cattle have been frequently analysed, the physiological differences underlying them have not been widely studied. The hormonal control of energy partition and its possible effects on milk yield and other production traits has been reviewed above (see Introduction, section I.2.3). The evidence indicated that insulin, growth hormone, thyroxine and triiodothyronine were all involved in the control of energy metabolism. The present experiment was designed to study these four hormones in two groups of animals known to differ in genetic merit for milk production : these were a group of purebred Friesian and a group of Hereford x Friesian crossbred bull calves. The comparison was made between rather than within breeds partly because of the larger expected differences in energy partition, and partly because of the difficulty of obtaining young dairy bull calves, matched for age, of sufficiently different genetic backgrounds (for milk production) to enable comparison.

The object of the experiment was to estimate, for each of the four hormones measured :

- 1) the extent of diurnal variation
- 2) the repeatability of measurement

- 3) the extent of prandial variation, and possible responses to fasting, refeeding and injection of energy metabolites
- 4) breed differences in patterns of secretion or absolute levels
- 5) relationships with the other hormones measured or with growth rate or body weight.

The experiment was performed in two parts in order to study the energy metabolism of the calves first (I) in the pre-rumination, monogastric state and later (II) when rumination was fully established.

The injection of energy metabolites was performed chiefly for their known stimulatory effect on insulin secretion (McAtee and Trenkle, 1971). The use of such stimuli might reveal or magnify relevant differences between breeds or between animals in their patterns of hormone secretion. The choice of glucose and sodium propionate was made for the following reasons. The digestive system of preweaning calves is similar to other monogastric mammals in that glucose, being an important end-product of carbohydrate digestion, is absorbed from the gut in considerable quantities. When rumination becomes established postweaning, the ruminal bacteria ferment glucose further, little glucose is absorbed from the gut and most has to be synthesized by the animal from glycogenic precursors of which propionate is probably the most important (Thatcher et al., 1977). The two substances, therefore, probably play important roles in preweaning and postweaning energy metabolism, and both are known to stimulate insulin secretion in postweaning cattle (McAtee and Trenkle, 1971) and sheep (Ambo, Takahashi and Tsuda, 1973).

The doses of glucose and propionate used for injection were based on those used by McAtee and Trenkle (1971), which in turn were based on the rates of production of fatty acids in the rumen (Stewart, Stewart and Schultz, 1958).

V.2 Materials and methods.

V.2.1 Animals.

12 entire male calves, all born in the same 7-day period, were purchased when under 14 days old. Six were British Friesian and six Hereford x Friesian crosses. They were housed indoors throughout the experimental period and bedded on wood shavings. All animals were weighed weekly and their rations adjusted accordingly. Up to the end of part I of the experiment they were fed entirely on powdered milk (Volac; Royston, Herts) made up with water at the rate of 12% of body weight per day in three equal feeds, one at 0815, one at 1215 and one at 1615 hours. Between the ages of 7 and 12 weeks the calves were gradually weaned on to a pelleted complete diet AA6 containing 30% straw (Wainman et al., 1975) which was then fed ad libitum. One Friesian calf died between parts I and II of the experiment and was replaced by another Friesian calf approximately one week older but about 15 kg heavier than the remaining animals.

V.2.2 Experimental procedure : part I (calves = 6 weeks ⁺ 3 days old).

A cannula was placed in the jugular vein of each calf one day before the start of the experiment. The cannula was filled with heparinized isotonic saline to prevent clotting. The procedure for taking blood samples was as follows; all saline was withdrawn from the cannula, 10 mls of blood were taken into a syringe, and the cannula was refilled with sterile heparinized saline. The blood was centrifuged in a heparinized tube, the plasma removed and stored frozen at - 20°C. A total of 48

samples was taken from each calf. For each of the four hormones measured, these samples were assayed by the methods described (see chapter II, Assay Methods) in two assays of 24 samples each, in randomized sequence.

The experimental schedule was as follows (see Figure 5.1) :

Day 1 : Blood samples were taken hourly for 24 hours starting at 0700 hrs.

Day 2 : No morning or midday feed given. Samples were taken 2-hourly from 0800 to 1600 hrs. The animals were then fed and sampled hourly until 2100.

Day 3 : No samples, normal feed.

Day 4 : No morning feed given. Six calves chosen at random (3 per breed) received an injection of sodium propionate and the other six an injection of glucose at 0900 hrs. Samples were taken at -60, 0, 20, 40, 60, 90 and 120 minutes after the injection. The doses of propionate and glucose were 0.5 mmole/kg body weight, administered via the cannula at a concentration of 2.5 mol/l, pH 7.4. The calves were fed at 1215 and 1615 hours.

Day 5 : No morning feed. The same procedure was followed as for day 4 except that the calves which had had glucose injections received propionate and vice versa. The animals were fed at 1215 and the cannulae were then removed.

V.2.3 Experimental procedure : part II (calves = 16 weeks old).

The experimental procedure followed a similar pattern to part I with 24 hourly samples followed by a fasting period, refeeding, and injections

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S = blood sample (10 mls)

I = injection of glucose or propionate

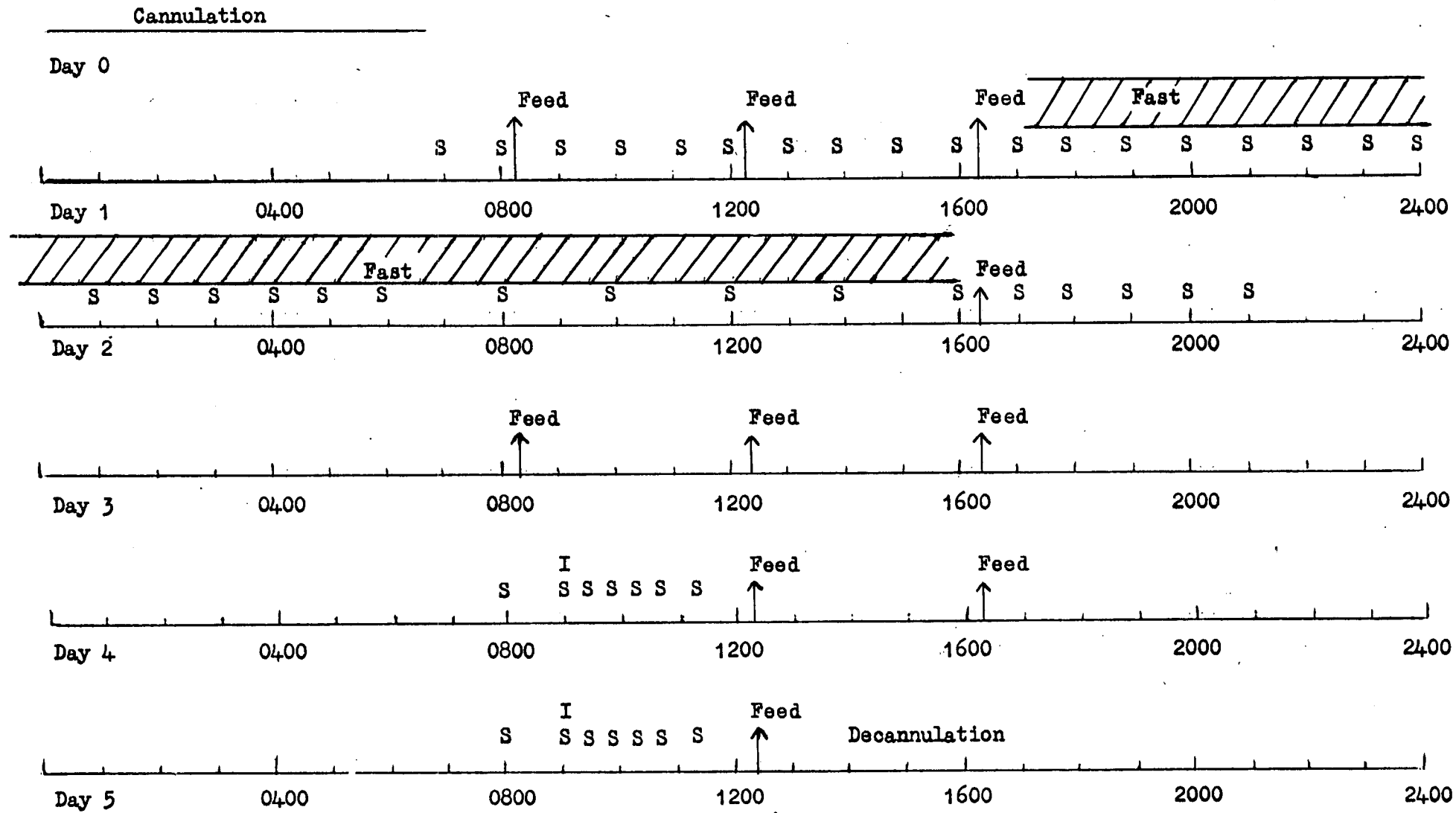


Figure 5.1

of energy metabolites. Methods used for cannulation and blood sampling were the same as for part I. The main differences were :

Concentrates were available ad libitum except where stated otherwise. The fasting period was extended to 48 hours, but with less frequent sampling, because the process of rumination causes a longer delay between the ingestion of food and the absorption of nutrients from the gut, thus buffering the effect of fasting.

For the injection of energy metabolites, glucose was not used because of its reduced importance in postweaning metabolism (see section V.1). Instead, two doses of propionate were used at three different times after withdrawal of blood. Insulin levels are known to fall with fasting (Hove and Blom, 1973; McAtee and Trenkle, 1971) and the responses to the injections might therefore be expected to occur against different background levels. In order to avoid bias in statistical analysis of responses, one animal in each breed was allocated to each of the following treatments for the injections (H = high dose, L = low dose) : HHL, HLH, HLL, LLH, LHL, LHH. The two doses were 0.5 and 1.0 mmole/kg body weight.

The experiment schedule was as follows (see Figures 5.2 and 5.3) :

Day 1 : Blood samples were taken hourly for 24 hours from 0700 hours.

Day 2 : Food was withdrawn after the sample at 0600 until day 4. Samples were taken 4-hourly.

Day 3 : Blood samples taken 4-hourly, No food given.

Day 4 : Food was replaced at 0900. Samples were taken hourly until 1600.

The animals were decannulated, rested on days 5 and 6 and recannulated on day 7.

Day 8 : Food was withdrawn at 0900. Injection of propionate was given at 1200. For this injection and for those on the two subsequent days, blood samples were taken at times -60, 0, 20, 40, 60, 90 and 120 minutes after the injection.

Day 9 : No food given. Injection of propionate was given at 0900, 24 hours after food withdrawal. Blood samples were taken as described for day 8.

Day 10 : No food given. Injection of propionate was given at 0900, 48 hours after food withdrawal. Blood samples were taken as described for day 8. After the final sample food was replaced and the animals were decannulated.

A total of 67 samples was taken from each animal in part II of the experiment. For each of the four hormones measured, three assays were performed, the first containing the first 24 samples, the second containing the next 22 samples and the third containing the last 21 samples, all in randomized sequence within the assays.

V.2.4 Statistical methods.

The following methods apply to both part I and part II of the experiment unless stated otherwise.

V.2.4.1 Hourly samples for 24 hours.

For each hormone the mean levels of the six animals in each breed were calculated and plotted against time. The difference between the two means at each sample time was tested for significance on a t-test.

Experimental schedule for hormone measurements in dairy and crossbred calves at 16 weeks of age.

S = blood sample (10 mls)

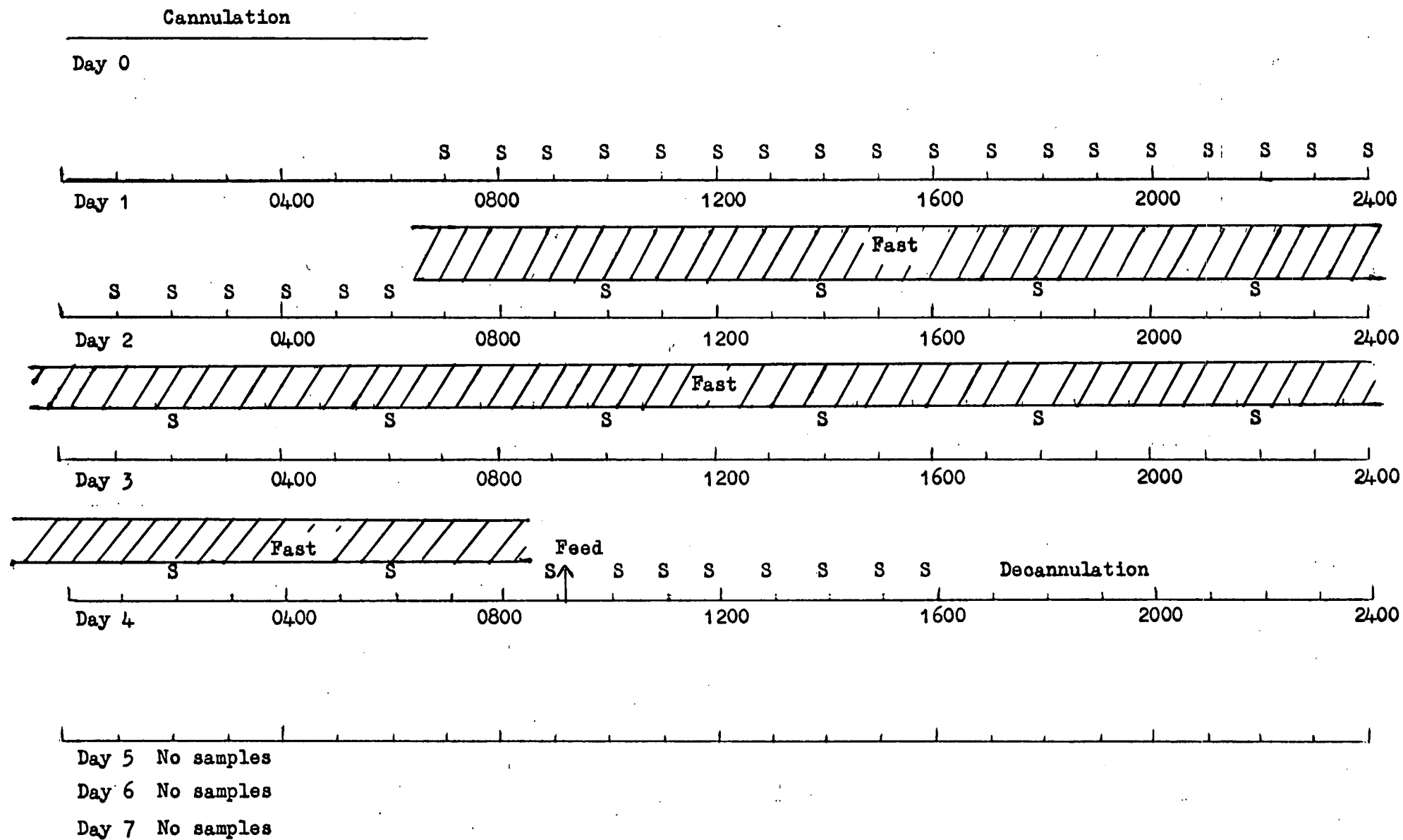


Figure 5.2

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(continued from Figure 5.2)

S = blood sample (10 mls)

I = injection of propionate

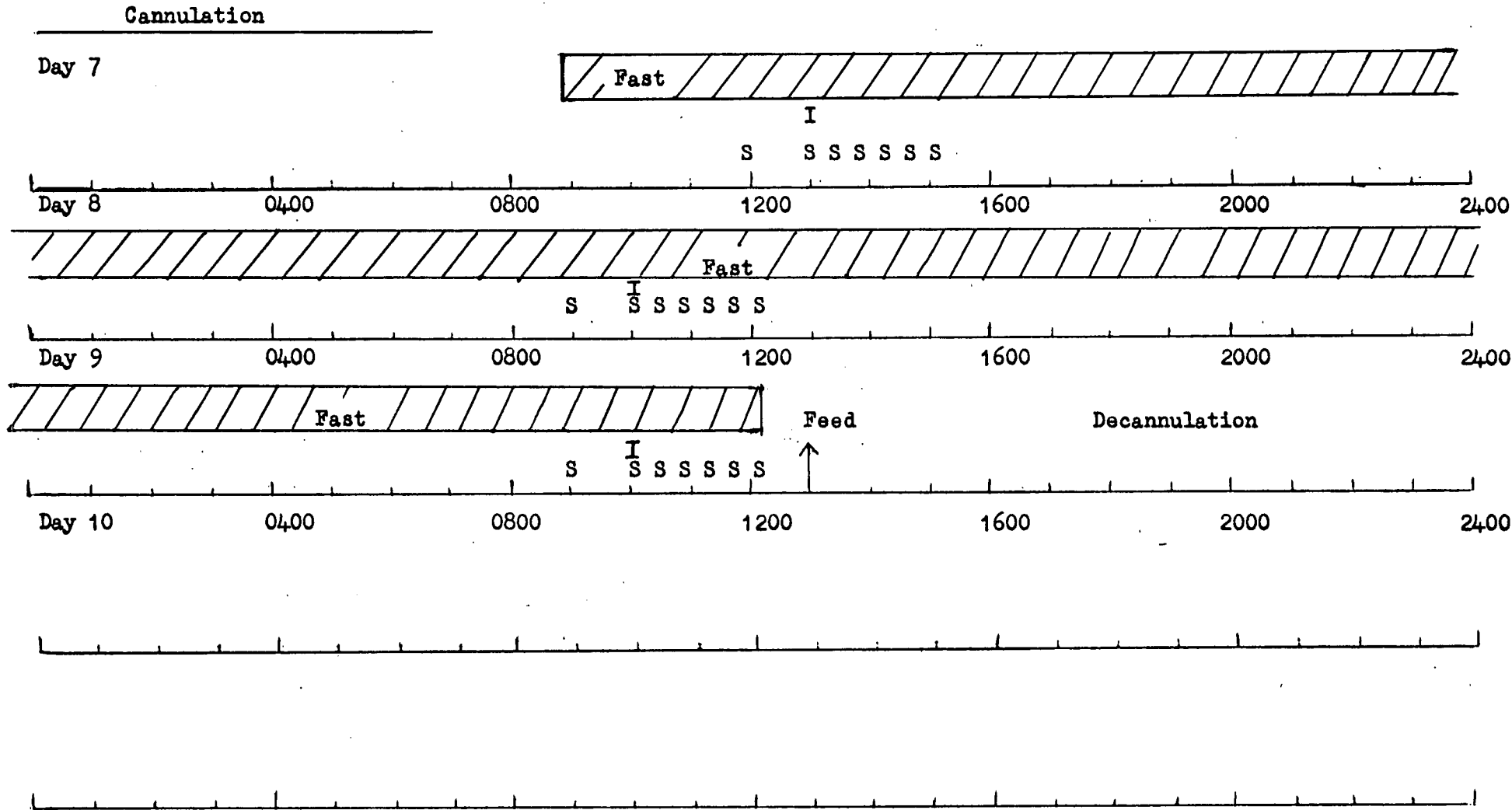


Figure 5.3

For T3, T4 and growth hormone, missing values (due to missing samples or failed assay) occurred at two or less of the 24 sample times. In order to avoid unequal subclass numbers, such times were omitted when the results for the relevant hormone were analysed, namely : part I, T3 2400 hrs, T4 1100 and 2400 hrs; part II, T3 0300 hrs, T4 0700 hrs. An analysis of variance was performed for each hormone and the variance due to breeds, animals within breeds and the residual variance were calculated. The breed mean square was tested for significance against the animals mean square, and the animals mean square against the residual. Components of variance between and within animals were calculated and the repeatability estimated.

For insulin, missing values occurred at several sample times. Also, preliminary results indicated that there might be a breed x time interaction. Analysis of insulin levels was therefore performed on the computer using Harvey's (1960) programme for least-squares analysis of variance. Two analyses were performed. In the first analysis the effects fitted were 1) mean 2) animals 3) time 4) breed x time interaction. In the second analysis the effects fitted were 1) mean 2) breed 3) time 4) breed x time interaction. The sum of squares between animals within breeds was obtained by subtracting the residual SS obtained in the first analysis from that obtained in the second analysis. Components of variance between and within animals and the repeatability were also computed.

V.2.4.2 Samples during fasting and refeeding.

Breed means for each hormone were calculated and plotted against sample time. For each hormone, mean levels at the start and at the end of the

fasting period were tested against each other both within and across breeds. Similarly the mean levels after fasting were tested against the mean levels after refeeding.

V.2.4.3 Samples before and after injection of energy metabolites.

Hormonal responses to the injections were examined for all animals individually in order to identify a consistent pattern. This process was repeated for all four hormones. Where no pattern was identified, mean levels after injection were tested against mean levels before injection, both within and across breeds. For the hormones where these tests were not significant at any sample time, no further analysis was carried out.

For the hormones where a response was observed, the response was defined and measured for each animal after each injection. The responses of the twelve animals were then analysed with respect to :

- (i) any breed difference in response to injections of energy metabolites
- (ii) their relationship to the responses of the same animals to the other injections
- (iii) their relationship to the responses to feeding (part I only)
- (iv) the effect of propionate dose on the response (part II only)
- (v) the effect of length of fasting on response (part II only)
- (vi) the relationship of these responses to body weight and growth rate.

V.2.4.4 Mean hormone levels and correlations with other traits.

The following procedure was followed in both parts I and II. For each hormone, a mean value was calculated for each animal as the mean of the 24 1-hourly samples on days 1-2. Body weight was measured and liveweight gain up to the experimental period from age 2 weeks was calculated. Within breed correlations among all these traits at both ages were computed.

V.3 Results.

V.3.1 Part I : 24 hourly samples from 6-week old calves.

The graphs of mean hormone levels for each breed plotted against time are shown for the four hormones in figures 5.4 to 5.7. The overall mean hormone levels for each animal and for the two breeds are given in table 5.2 and the corresponding analyses of variance in table 5.1. For T₄, T₃ and growth hormone (GH) there were no obvious time effects such as circadian or prandial rhythms and so all 24 samples from each animal were treated as replicates in the analysis.

Both T₄ and T₃ showed a consistent breed difference over the 24-hour period with the crossbred calves having higher levels than the dairy calves, but this difference was only significant on a t-test at a few sample times. For both hormones this breed difference approached significance on an F-test in the analysis of variance.

Growth hormone levels showed no consistent pattern of variation over the 24 hours and there was no apparent breed difference.

Insulin levels showed a marked rise in response to feeding after all three feeds, with the crossbred calves having an apparently greater response. Because of the variability among animals in the size of this response, the difference between the breed means was only significant shortly after feeding (at the points denoted by an asterisk in figure 5.4). These responses to feeding are considered further in section V.3.3 and table 5.3. In the analysis of variance (table 5.1), the effects of

sample time and the breed by time interaction were both statistically significant, although the breed effect did not quite attain significance at the 5% level.

Repeatability estimates for T3 and T4 were moderately high (0.55 and 0.45) whereas those for GH (0.17) and insulin (0.10) were low.

V.3.2 Part I : samples from 6-week old calves during fasting and refeeding.

The graphs of hormone levels against sample time during the period of fasting and refeeding are shown in figures 5.4-5.7. Levels of T3, growth hormone and insulin all declined during the fasting period and rose again subsequent to refeeding. Overall mean levels for these three hormones at the end of the fasting period were tested against the levels before fasting and against the levels after the refeeding period. The differences were all significant on a t-test. T4 showed no marked change in response to fasting, but levels were possibly lower between samples 16 and 22 (2200-0400 hrs) than before or after that period. For the thyroid hormones, the crossbred calves continued to show higher levels at most times, while for insulin a substantial breed difference was evident in the response to refeeding, with the crossbred calves showing a large rise but the purebred Friesians showing none (see figure 5.7).

V.3.3 Part I : samples from 6-week old calves before and after injection of energy metabolites.

Neither the glucose nor the propionate injections had any effect on levels of T4, T3 or growth hormone in either breed on either day of treatment. Insulin levels showed a consistent rise in response to propionate injection but no apparent response to glucose.

The insulin response was short-lived, only being detectable in the sample 20 minutes after injection, but was statistically significant. Basal levels were defined as the mean for each animal of the two samples before the injection, and the response was defined as the difference between the level in the 20-minute sample and the basal level. The basal levels were low in all cases (corresponding to the low levels observed after overnight fasting on day 2) and the responses were also small in comparison to the large responses to feeding observed on day 1. The results are shown in table 5.3.

The difference in mean response between breeds was not significant, nor was there any difference due to day of treatment. Correlations were determined between the responses and body weight, growth rate, and mean hormone levels (see next section). None of these was significant except the correlation of the response with the mean insulin level ($r = 0.725$, $P < 0.01$).

The relationship between the insulin response to propionate and the insulin rise after meals was investigated. The responses to feeding were large and somewhat variable (table 5.3). Consequently it was decided to use a log transformation of these levels before the within-breed correlations were calculated (table 5.3). These correlations were all positive and reached significance in 3 of the 6 cases despite the few degrees of freedom (7 or 8 in each case).

V.3.4 Part I : correlations among hormones and other traits.

Mean hormone levels over the 24 1-hourly samples are shown for all twelve animals in table 5.2, as are the values for liveweight gain up

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at 6 weeks of age (preweaning).

Source	T3			T4			GH			Insulin		
	d.f.	MS	F	d.f.	MS	F	d.f.	MS	F	d.f.	MS	F
Breed	1	14.55	3.64 ⁺	1	13995.6	3.82 ⁺	1	370.6	0.43	1	12.7	4.33 ⁺
Animals/breed	10	4.00	29.38***	10	3665.8	19.23***	10	859.3	5.82***	10	2.93	4.35***
Times	-	-	-	-	-	-	-	-	-	23	3.55	5.27***
Breed x times	-	-	-	-	-	-	-	-	-	23	1.29	1.91*
Residual	264	0.136		252	190.6		276	147.7		218	0.674	
k	23			22			24			22.8		
σ^2_B	0.168			157.9			29.6			0.098		
Repeatability	0.552 \pm 0.01			0.453 \pm 0.01			0.167 \pm 0.02			0.128 \pm 0.004		
$(\sigma^2_B / \sigma^2_B + \sigma^2_W)$												

Note : σ^2_W = Residual MS, σ^2_B = (Animals MS - Residual MS)/k.

+ = P < 0.10

Table 5.2 :

Mean hormone levels over 24 hours together with body weight and liveweight gain in 12 calves at 6 weeks of age (preweaning).

F = Friesian, H F = Hereford x Friesian

Animal	T ₄ (ng/ml)	T ₃ (ng/ml)	Insulin (ng/ml)	GH (ng/ml)	Body wt. (kg)	Gain (kg) 2-6 wks	Gain (kg) 2-16 wks
F1	30.2	0.83	0.417	24.2	47.2	6.3	47.6
F2	48.4	1.09	0.172	28.3	44.4	2.7	40.8
F3	11.7	0.27	0.089	40.1	34.5	-2.7	-
F4	15.2	0.45	0.502	30.4	39.5	0.5	32.6
F5	19.7	0.41	0.286	19.3	41.7	0.9	39.0
F6	27.5	1.06	0.213	26.8	54.0	7.7	54.0
H F1	55.0	1.35	1.172	27.9	38.5	7.3	46.3
H F2	56.5	1.91	0.408	26.4	36.3	2.3	36.7
H F3	35.0	1.31	0.536	37.5	43.5	8.6	48.5
H F4	26.8	0.73	0.448	30.4	40.4	9.1	54.9
H F5	27.5	0.66	1.433	35.2	37.6	2.7	54.0
H F6	26.7	0.84	0.285	25.6	38.5	6.3	48.1
Mean							
Friesian	23.2	0.68	0.280	28.2	43.6	2.6	42.8
H F	37.0	1.13	0.713	30.5	39.1	6.1	48.1
Overall Mean	30.2	0.90	0.496	29.4	41.4	4.3	45.4

Table 5.3 : Insulin levels in 6-week old calves (preweaning) : insulin response to injection of sodium propionate and insulin levels after each of 3 feeds : correlations among these levels.

F = Friesian, H F = Hereford x Friesian

Insulin level	H F1	H F2	H F3	H F4	H F5	H F6	F1	F2	F3	F4	F5	F6
Pre-injection	0.033	0.039	0.067	0.027	0.091	0.064	0.035	0.075	0.008	-	0.015	0.012
Post-injection	0.217	0.090	0.171	0.047	0.866	0.181	0.359	0.088	0.027	-	0.600	0.108
Response	0.184	0.061	0.104	0.020	0.775	0.117	0.324	0.013	0.019	-	0.585	0.096
After feed 1 (0900 hrs)	11.658	2.144	0.780	0.248	7.824	1.353	1.350	0.396	0.513	1.798	-	0.388
After feed 2 (1300 hrs)	2.094	1.097	0.896	1.31	1.643	0.306	1.198	0.152	0.060	1.277	0.803	0.194
After feed 3 (1700 hrs)	1.683	1.421	1.198	-	2.450	0.610	1.083	0.905	0.027	0.595	-	0.832

Within-breed correlations among log insulin levels :-

	A)	B)	C)	D)
A) After propionate	1.00			
B) 0900 hrs	0.72*	1.00		
C) 1300 hrs	0.67*	0.52	1.00	
D) 1700 hrs	0.46	0.29	0.68*	1.00

Figure 5.4 :

Mean plasma T3 levels in 6 dairy and 6 crossbred bull calves at 6 weeks of age.

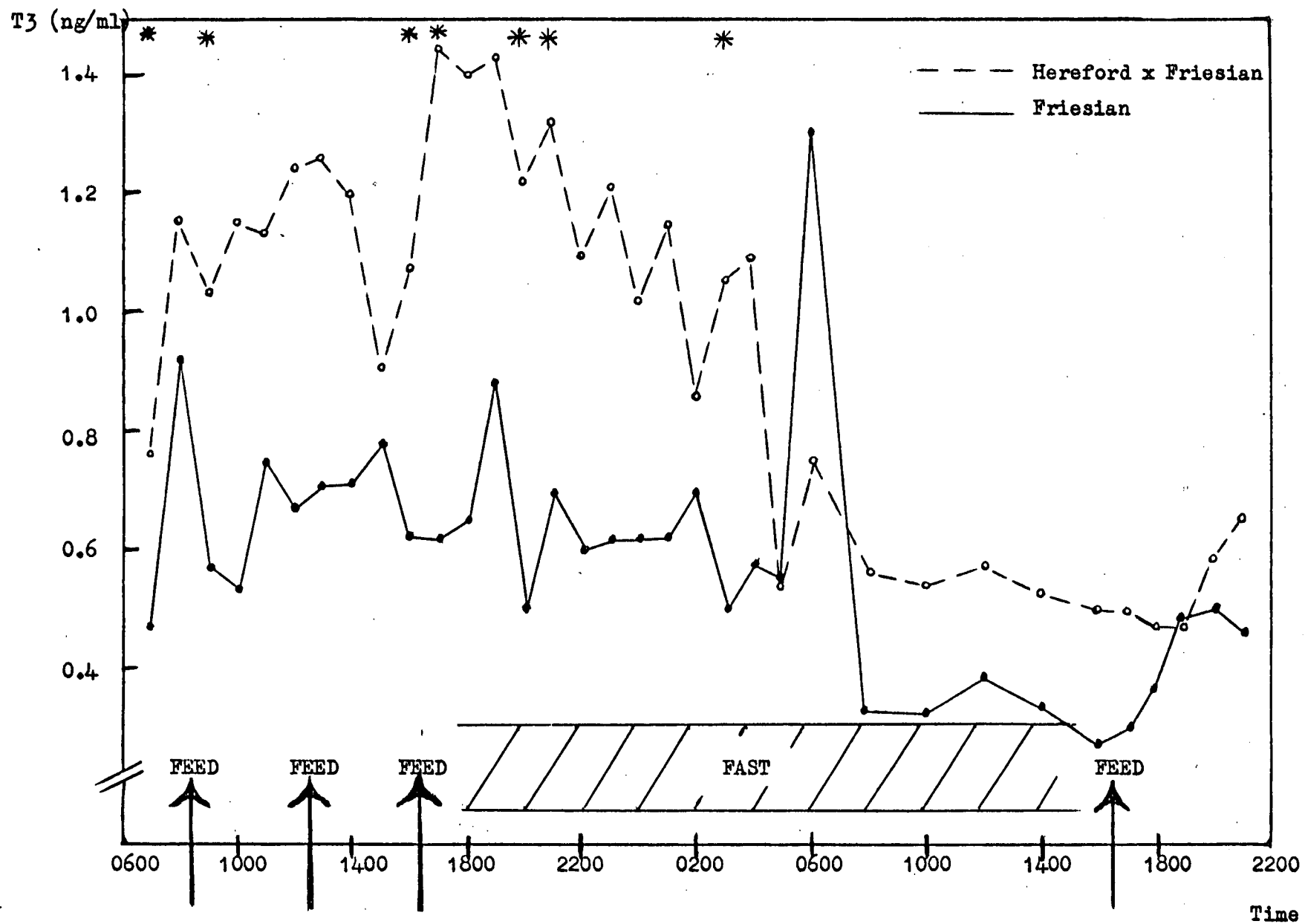


Figure 5.5 :

Mean plasma T4 levels in 6 dairy and 6 crossbred calves at 6 weeks of age.

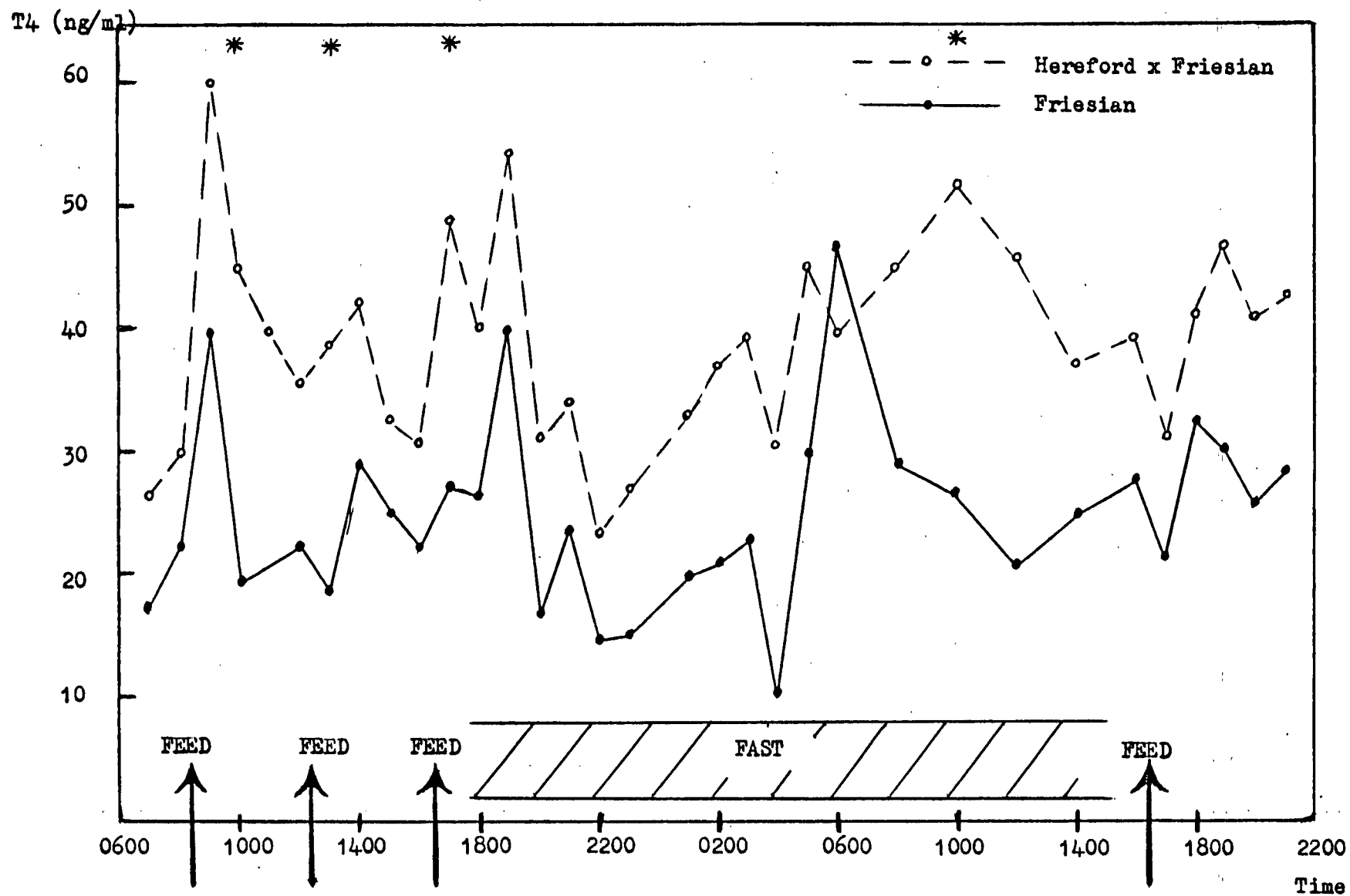


Figure 5.6 :

Mean plasma growth hormone levels in 6 dairy and 6 crossbred calves at 6 weeks of age.

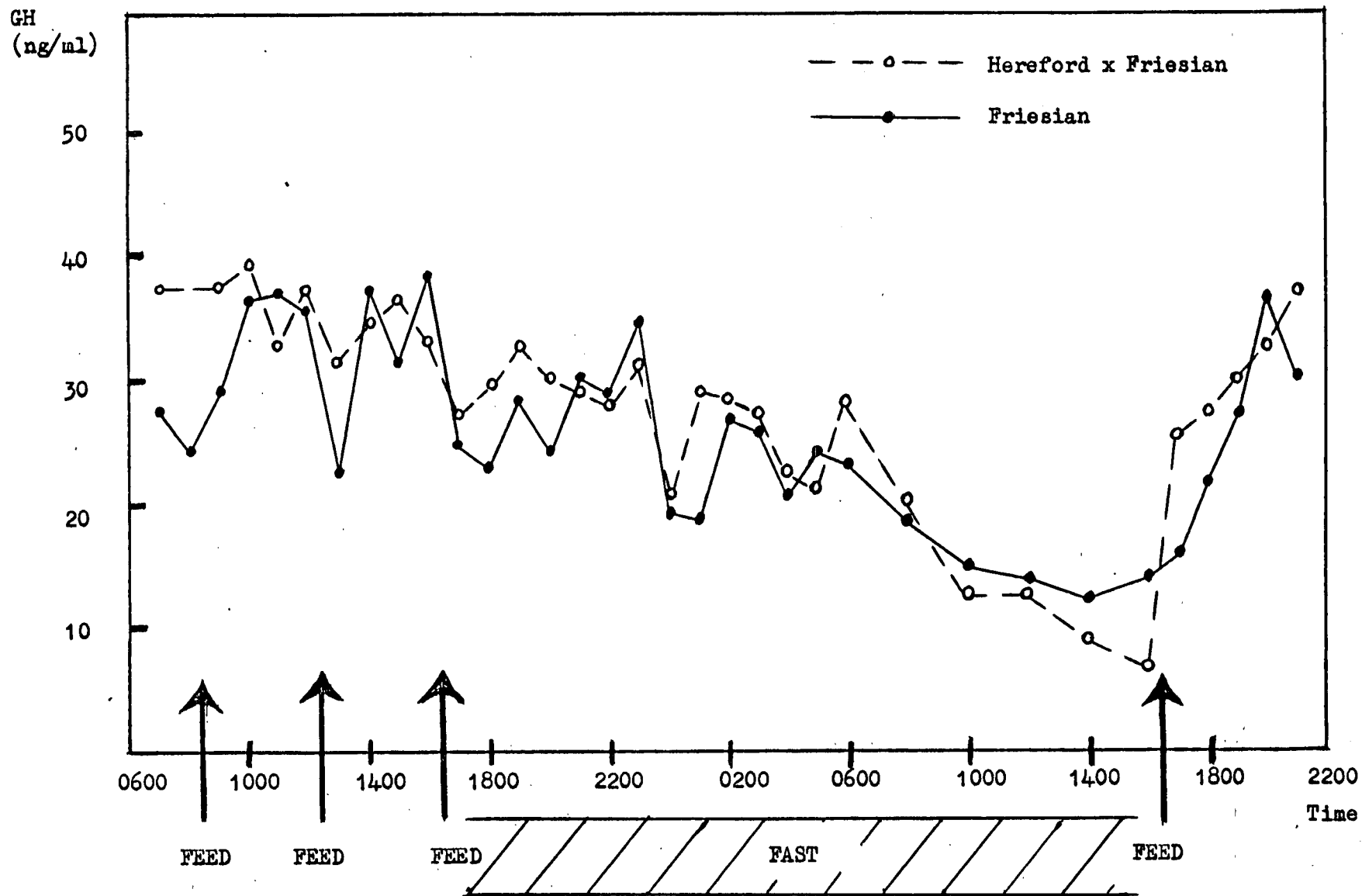
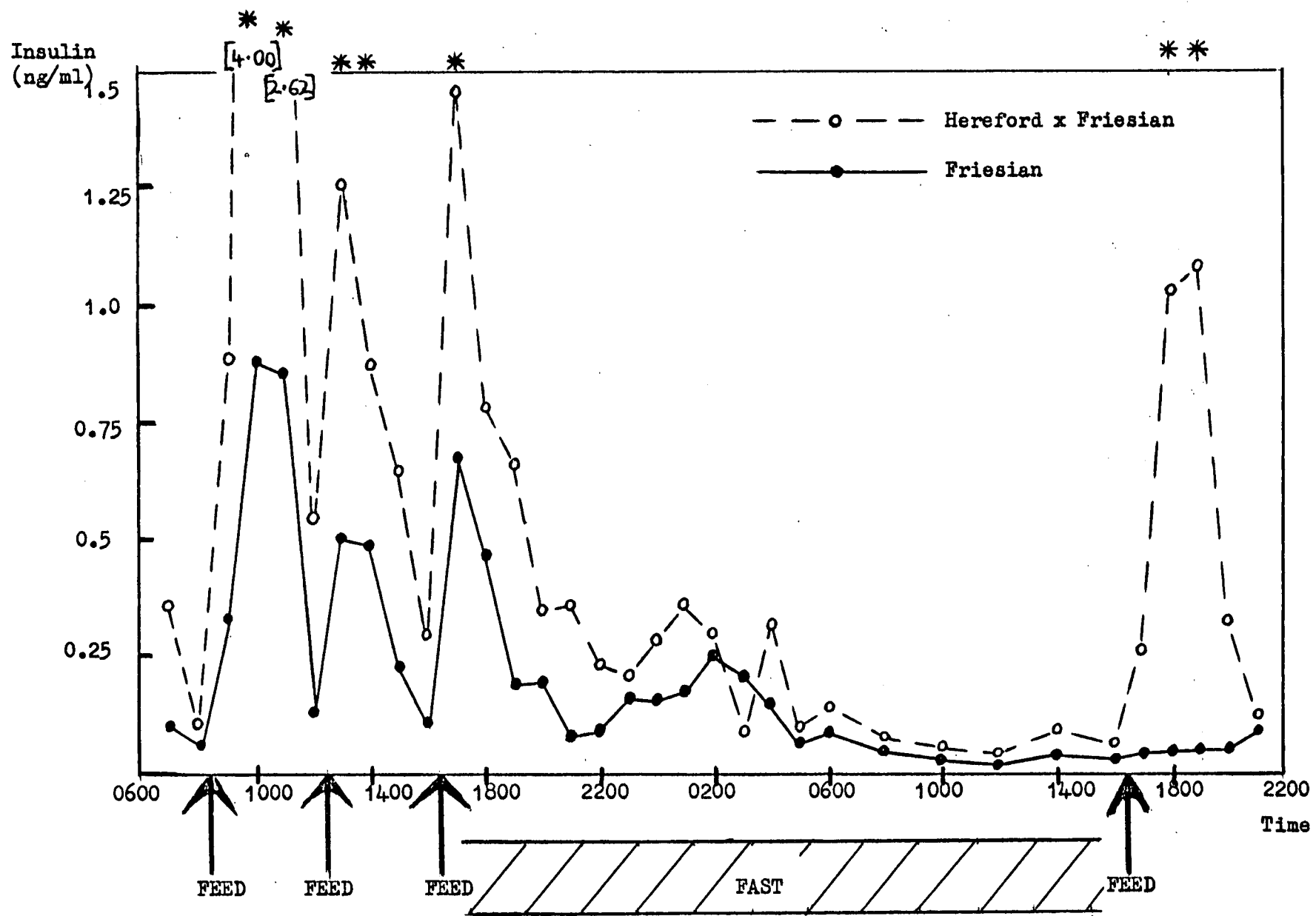


Figure 5.7 :

Mean plasma insulin levels in six dairy and six crossbred bull calves at 6 weeks of age.



to the experimental period and body weight. It can be seen that animal number F3 was the lightest, had lost weight and showed reduced levels of T4, T3 and insulin although its GH level was elevated. This calf later died.

Within breed correlations among all the traits measured at age 6 weeks are shown in table 5.9. Mean levels of T4 and T3 were highly correlated ($r = 0.859$). Also, there was a within-breed correlation between the animals' insulin response to propionate and their mean 'basal' level of insulin over the 24-hour period ($r = 0.725$).

V.3.5 Part II : 24 hourly samples from 16-week old calves.

The graphs of mean hormone levels against sample time are plotted for each breed in figures 5.8-5.11. The corresponding analyses of variance are shown in table 5.4. The overall mean levels for each animal and for the two breeds are shown in table 5.5.

Mean levels of T4 and T3 at individual times of sampling tended to resemble each other over the 24-hour period, even though no consistent diurnal pattern was apparent. Although the crossbred calves tended to have higher levels both of T4 and T3 than the Friesians, this difference was smaller than in part I and over the whole 24 hours was non-significant on an F-test.

Growth hormone levels rose sharply at the start of the sampling period (samples 1-5) and did not return to the initial low levels even after 24 hours. The tendency for the crossbred calves to have higher levels was not significant.

Insulin levels in both breeds were elevated between samples 10 and 17 (1700-2400 hours) and the effect of sample time was correspondingly significant in the analysis of variance. It is possible that this rise may be caused by absorption of nutrients from the gut, comparable to the rises in insulin after meals in part I. The insulin response to refeeding after fasting tended to confirm this, although there was a time lag between the replacement of food and the insulin response. The reasons for this are discussed in the Discussion.

As in part I, repeatability estimates for T3 and T4 were moderately high (0.68 and 0.58) but estimates for insulin and GH were only 0.20 and 0.06 respectively.

V.3.6 Part II : samples from 16-week old calves during fasting and refeeding.

Figures 5.8-5.11 show the graphs of hormone levels against time during the period of fasting and refeeding. Overall mean levels of all four hormones declined during fasting; insulin and GH then rose again significantly while T3 and T4 did not. The rise in GH appeared to begin 2 hours after the replacement of food but the rise in insulin did not occur until about 1900 hrs, 5 hours after the replacement of food.

V.3.7 Part II : samples from 16-week old calves before and after injection of an energy metabolite (sodium propionate).

Injection of propionate had no significant effect on levels of T4 or T3 in either breed at either dose at any of the three sample times. Insulin

levels rose in response to injection, as in part I, and GH levels fell : both these responses were most noticeable on the first occasion. The responses for both hormones were defined as the absolute difference between the level in the 20-minute sample and the mean level in the previous two samples. The mean responses for each breed-dose-time combination are shown in table 5.6. The results of the analyses of variance for the responses are shown in tables 5.7 and 5.8.

The mean insulin response was consistently higher in the crossbred than in the dairy calves, particularly at the first time of injection, so that the breed effect and the breed x time interaction approached significance. The high propionate dose produced a consistently higher mean insulin response than the low dose and the effect was significant in the analysis. The effect of time was also highly significant with the size of response diminishing at the second and third times of injection.

The response of growth hormone to the propionate injections was more variable and the effects less clear-cut. Levels of growth hormone were depressed in both breeds at the first time; at the second time, only the low dose produced a depression in levels, while at the third time the low dose produced a marked rise in levels. The effect of time and the dose x time interaction were therefore significant. The significant breed x dose interaction was probably also due to the rise in levels for the Friesians on the low dose at time 3. Other effects, i.e. breed, dose, animals and breed x time were non-significant.

Least-squares mean responses were obtained for each animal after adjusting for dose and time. Within-breed correlations were calculated between these mean responses and body weight, growth rate and mean hormone levels (see next section). None were significant except the correlation of the insulin response with the mean insulin level over the 24 hour period ($r = 0.580$). The GH response was negatively correlated with mean GH level ($r = - 0.208$).

V.3.8 Part II : correlations among hormone levels and other traits.

Mean hormone levels over the 24 hourly samples are shown for each animal in table 5.5, together with body weight and weight gain. The replacement calf, no. F3, was considerably heavier than the others and was excluded from correlations involving body weight or weight gain to avoid bias.

Correlations among the traits at age 16 weeks are shown in table 5.10. Both T4 and T3 showed significant positive correlations with weight gain and with body weight. As in part I, they were strongly correlated with each other. In addition, there was a strong negative relationship between insulin and growth hormone levels.

Finally, the correlations between the traits at 6 weeks and the traits at 16 weeks were determined. These are shown in table 5.11. Levels of insulin were highly correlated between the two ages, apparently indicating a higher repeatability over age than during a day. Similarly, the correlation between the insulin responses to propionate at the two ages was positive though non-significant. Other significant correlations were between GH in part I and insulin part II, and between the thyroid

hormones in part I and GH in part II. Liveweight gains at the earlier and later stages were also correlated.

The relationship between growth hormone and body weight at 16 weeks was negative ($r = - 0.286$). If animal no. 7 was excluded (this animal was the lightest and somewhat weakly with the lowest levels of T₄, T₃ and GH) the correlation between GH and body weight became $- 0.741$.

Table 5.4 : Analysis of variance of hormone levels over 24 hourly samples in dairy and crossbred calves
at 16 weeks of age (postweaning).

Source	T3			T4			GH			Insulin		
	d.f.	MS	F	d.f.	MS	F	d.f.	MS	F	d.f.	MS	F
Breed	1	5.64	0.61	1	944.9	0.25	1	1720.4	2.87	1	0.0012	0.01
Animals/breed	10	9.24	50.88***	10	3811.4	32.99***	10	600.2	2.66**	10	0.1410	6.81***
Times	-	-	-	-	-	-	-	-	-	23	0.0857	4.14***
Breed x times	-	-	-	-	-	-	-	-	-	23	0.0242	1.17
Residual	264	0.184		264	115.5		276	225.9		221	0.0207	
k	23			23			24			23.1		
σ^2_B	0.394			160.7			15.6			0.0052		
Repeatability	0.684 ± 0.01			0.582 ± 0.01			0.065 ± 0.001			0.201 ± 0.007		
$(\sigma^2_B / \sigma^2_B + \sigma^2_W)$												

Table 5.5 :

Mean hormone levels over 24 hours together with body weight and liveweight gain in 12 calves at 16 weeks of age (postweaning).

F = Friesian, H F = Hereford x Friesian

Animal	T4 (ng/ml)	T3 (ng/ml)	Insulin (ng/ml)	GH (ng/ml)	Body wt. (kg)	Gain (kg) 2-16 wks
F1	30.7	0.94	0.196	33.2	88.4	47.5
F2	46.9	1.34	0.166	39.1	82.5	40.8
F3	55.4	1.45	0.262	35.8	127.9	-
F4	24.5	0.52	0.263	26.5	71.7	32.6
F5	34.5	1.40	0.132	33.5	79.8	39.0
F6	64.8	2.80	0.187	30.6	100.2	54.0
H F1	57.7	1.76	0.242	40.0	77.6	46.3
H F2	34.1	1.27	0.101	47.4	70.7	36.7
H F3	48.7	1.62	0.253	37.4	83.4	48.5
H F4	46.0	1.83	0.256	33.6	86.2	54.9
H F5	49.2	2.16	0.375	31.2	83.0	48.1
H F6	34.5	1.39	0.168	38.8	80.3	48.1
Mean						
Friesian	42.3	1.41	0.201	33.1	91.8	42.8
H F	46.0	1.67	0.232	38.1	80.2	47.1
Overall mean	44.2	1.54	0.216	35.6	86.0	44.9

Figure 5.8 :

Mean plasma T3 levels in 6 dairy and 6 crossbred bull calves at 16 weeks of age.

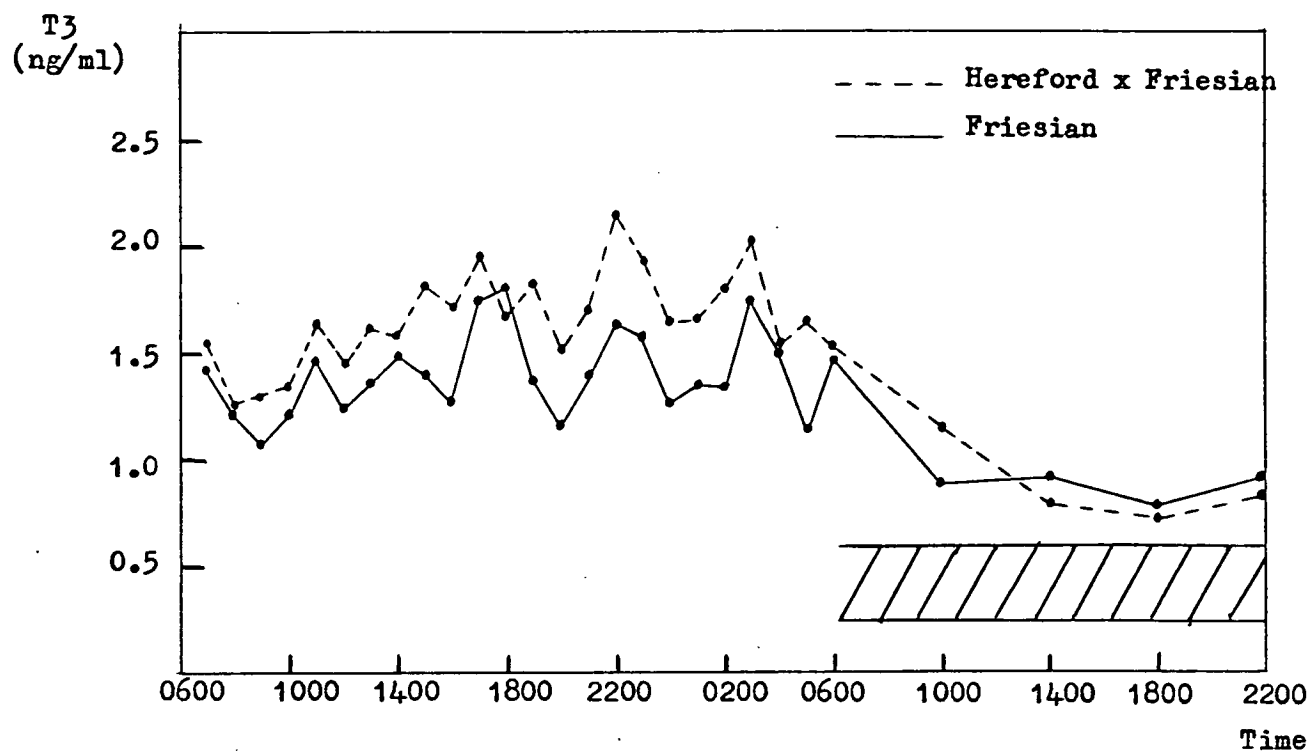


Figure 5.8 : continued

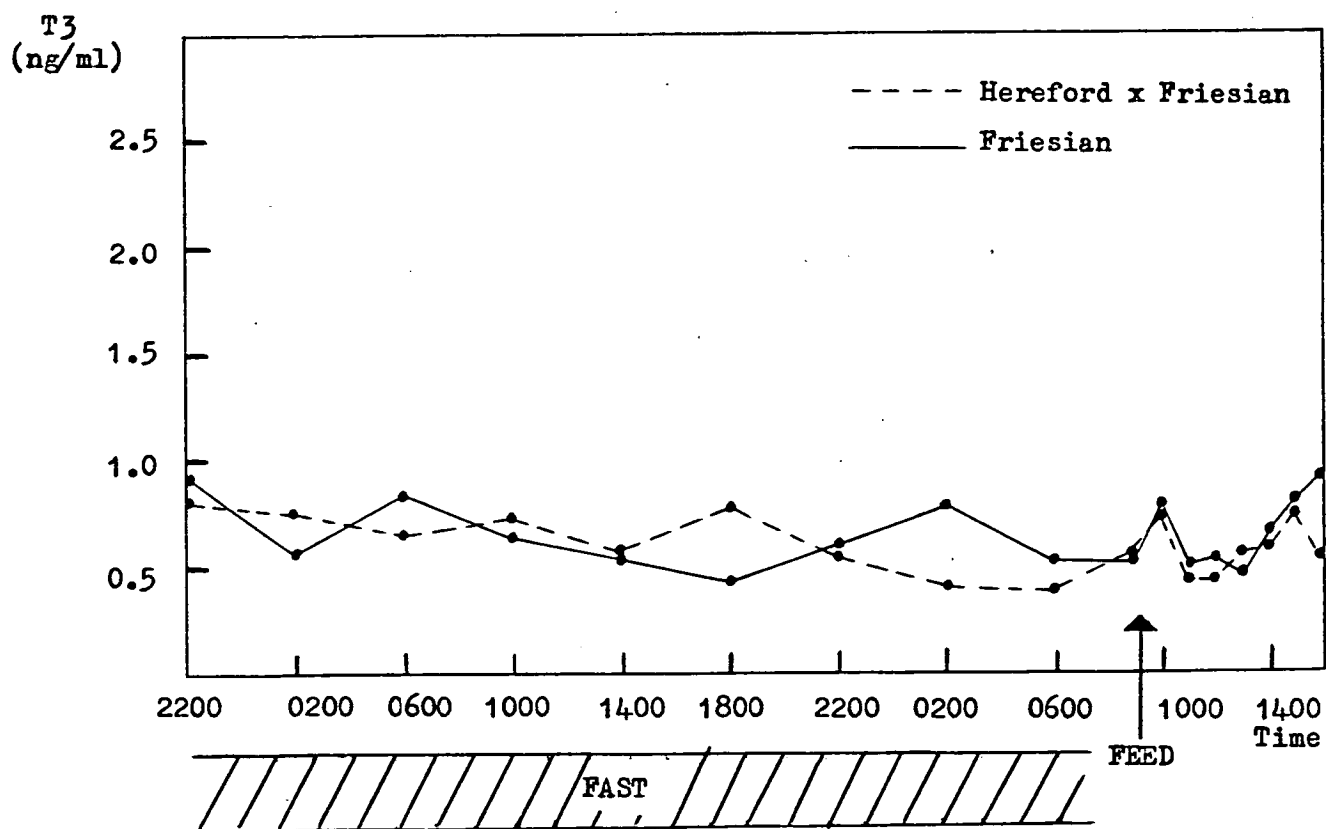


Figure 5.9 :

Mean plasma T₄ levels in 6 dairy and 6 crossbred steers at 16 weeks of age.

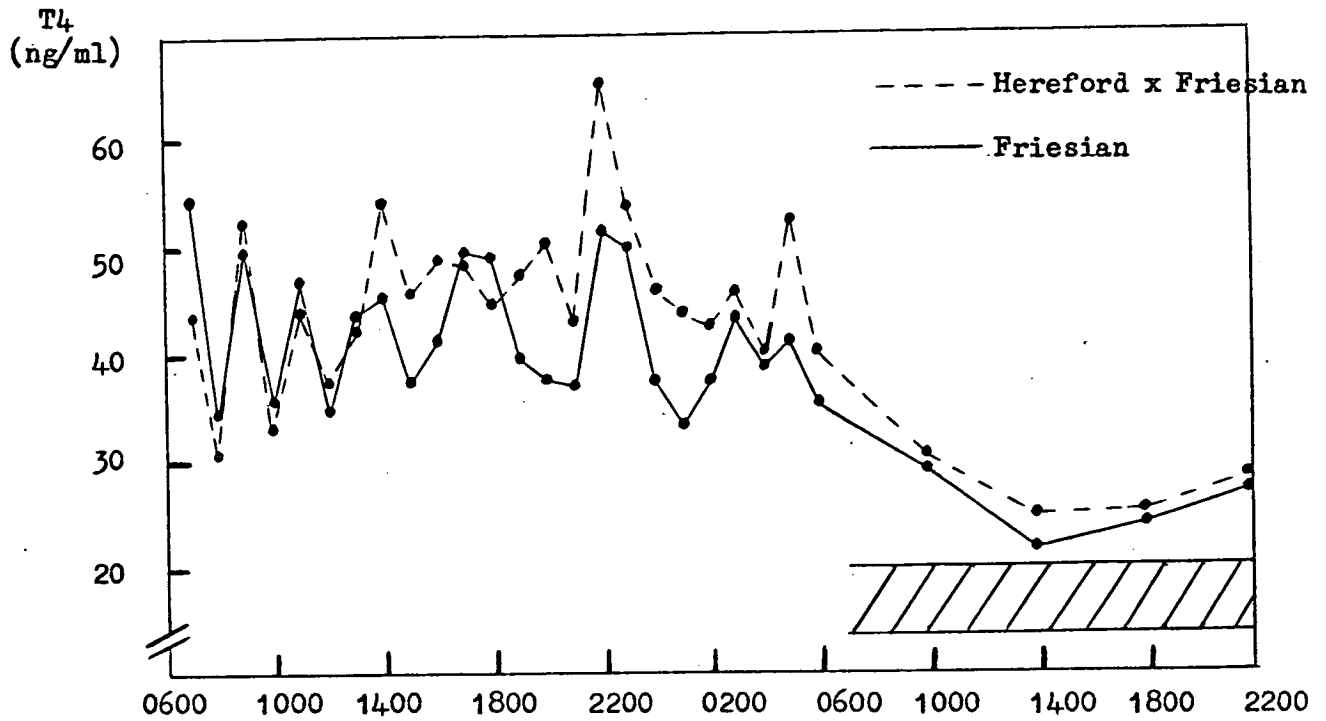


Figure 5.9 : continued.

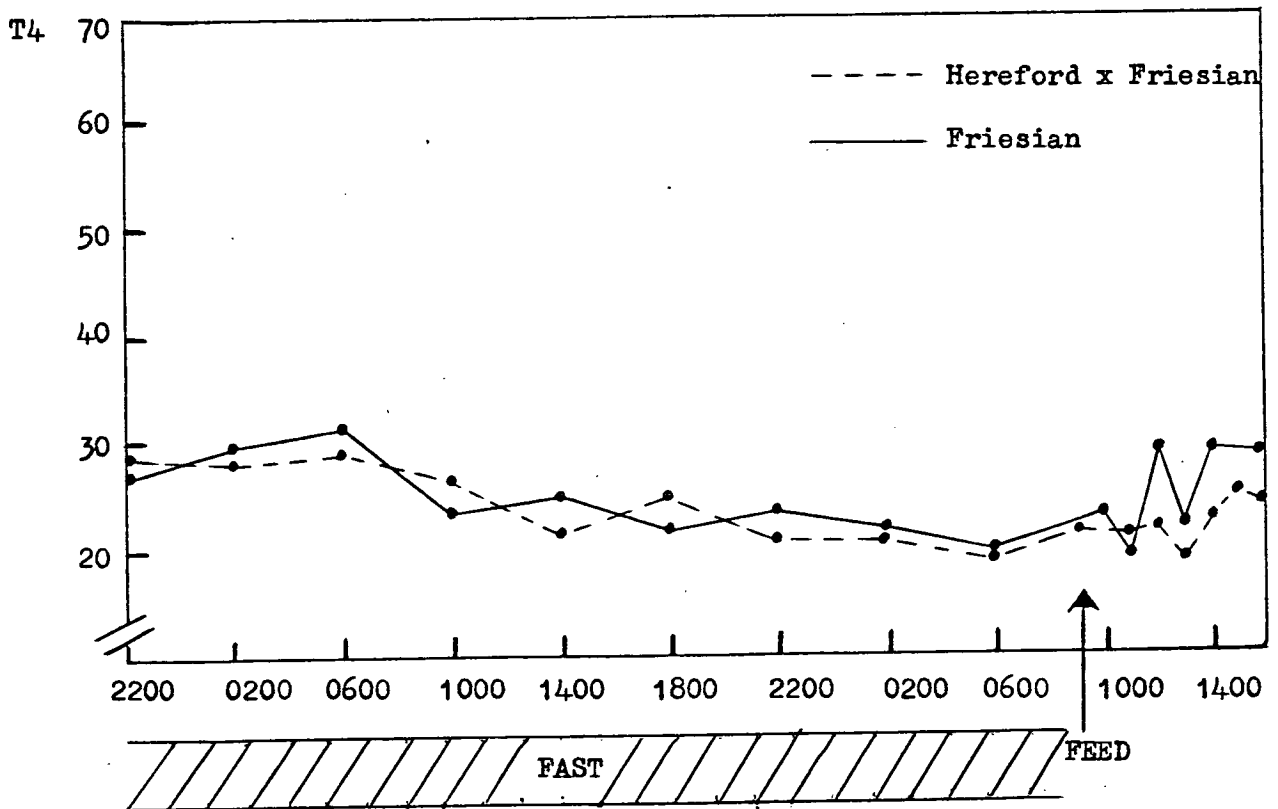


Figure 5.10 :

Mean plasma growth hormone levels in 6 dairy and 6 crossbred calves at 16 weeks of age.

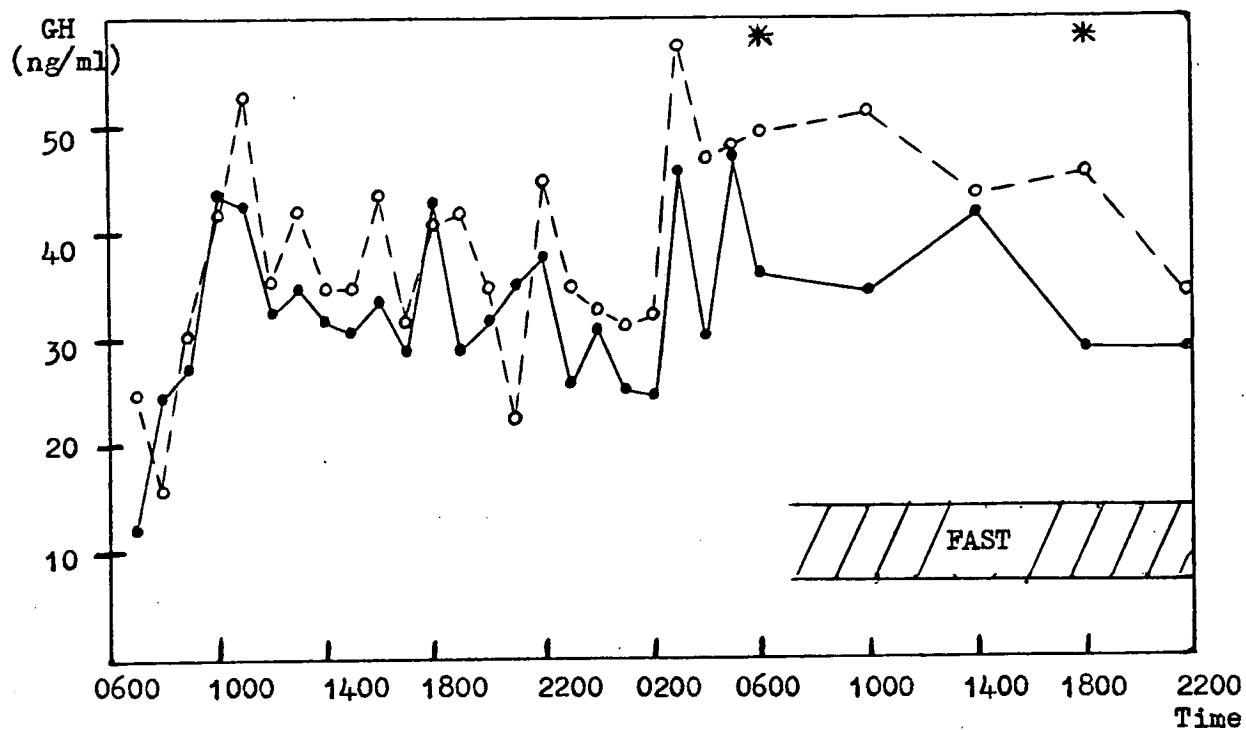


Figure 5.10 : continued.

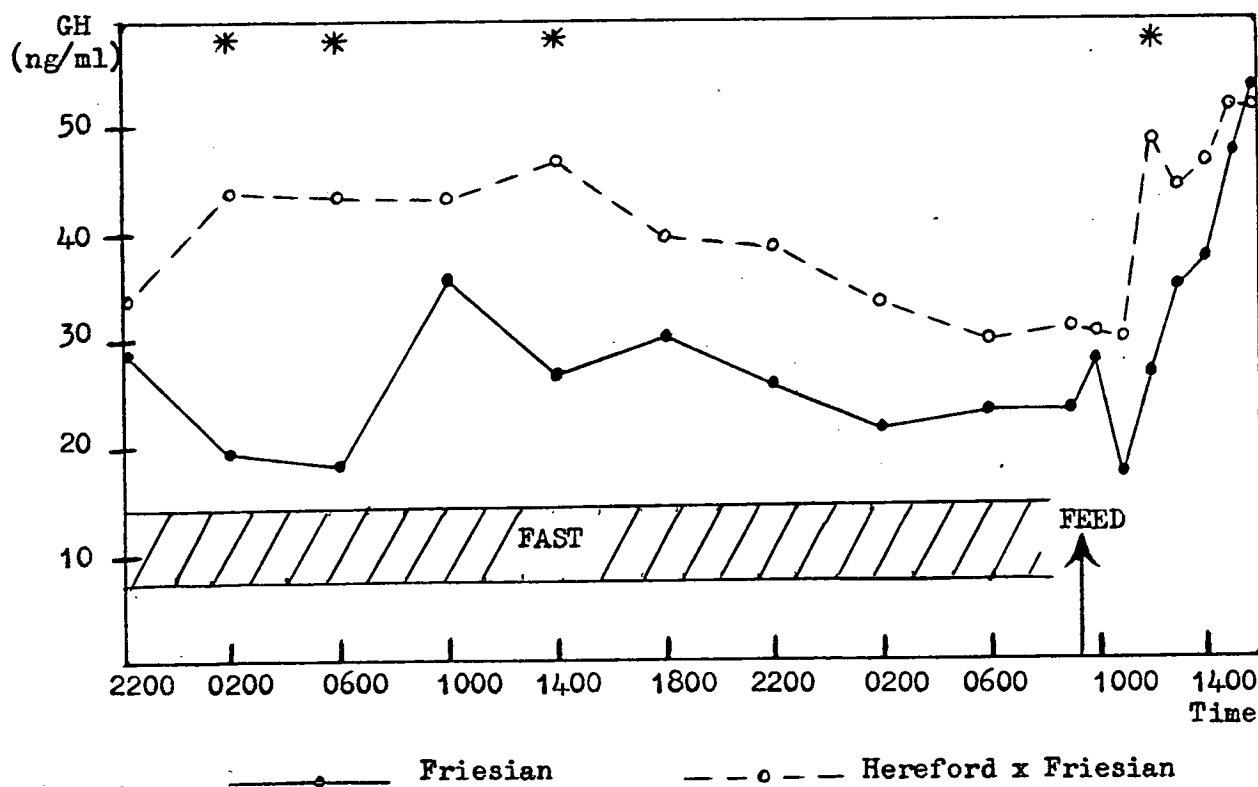


Figure 5.11 :

Mean plasma insulin levels in six dairy and six crossbred calves at 16 weeks of age.

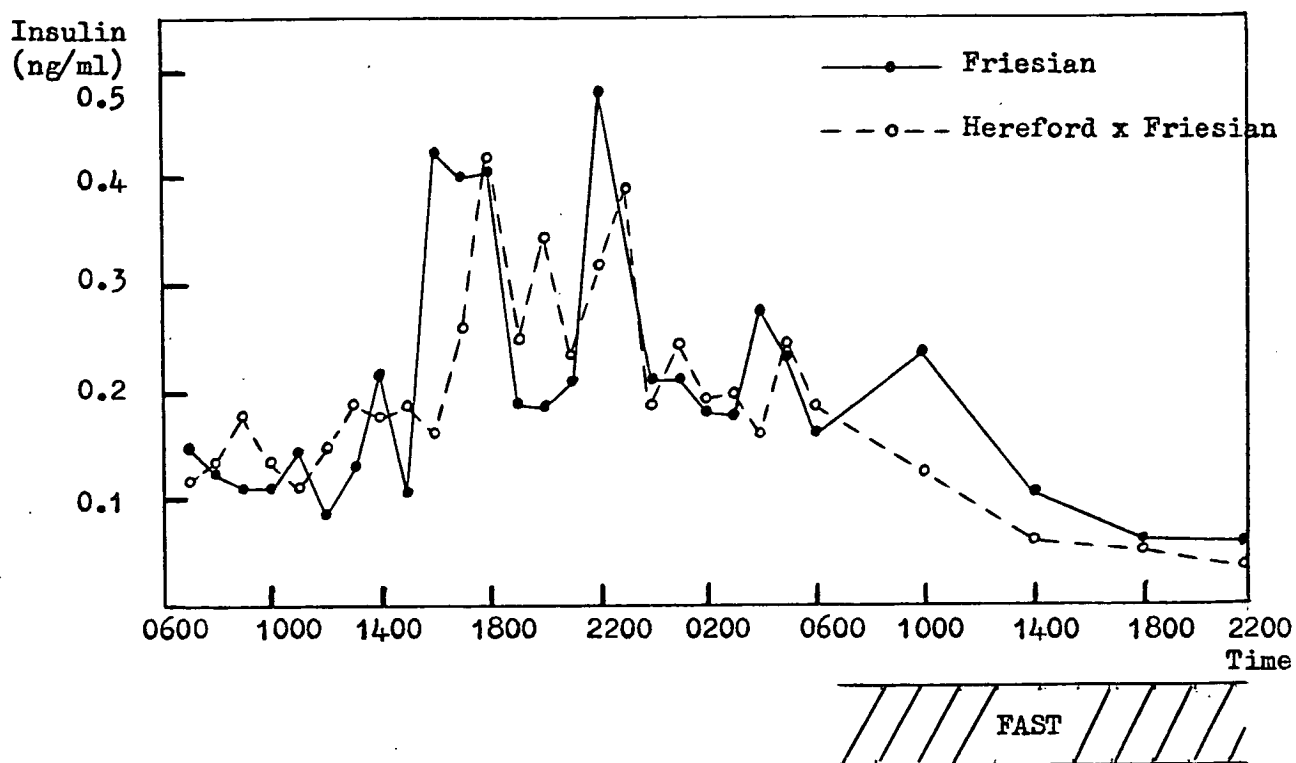


Figure 5.11 : continued.

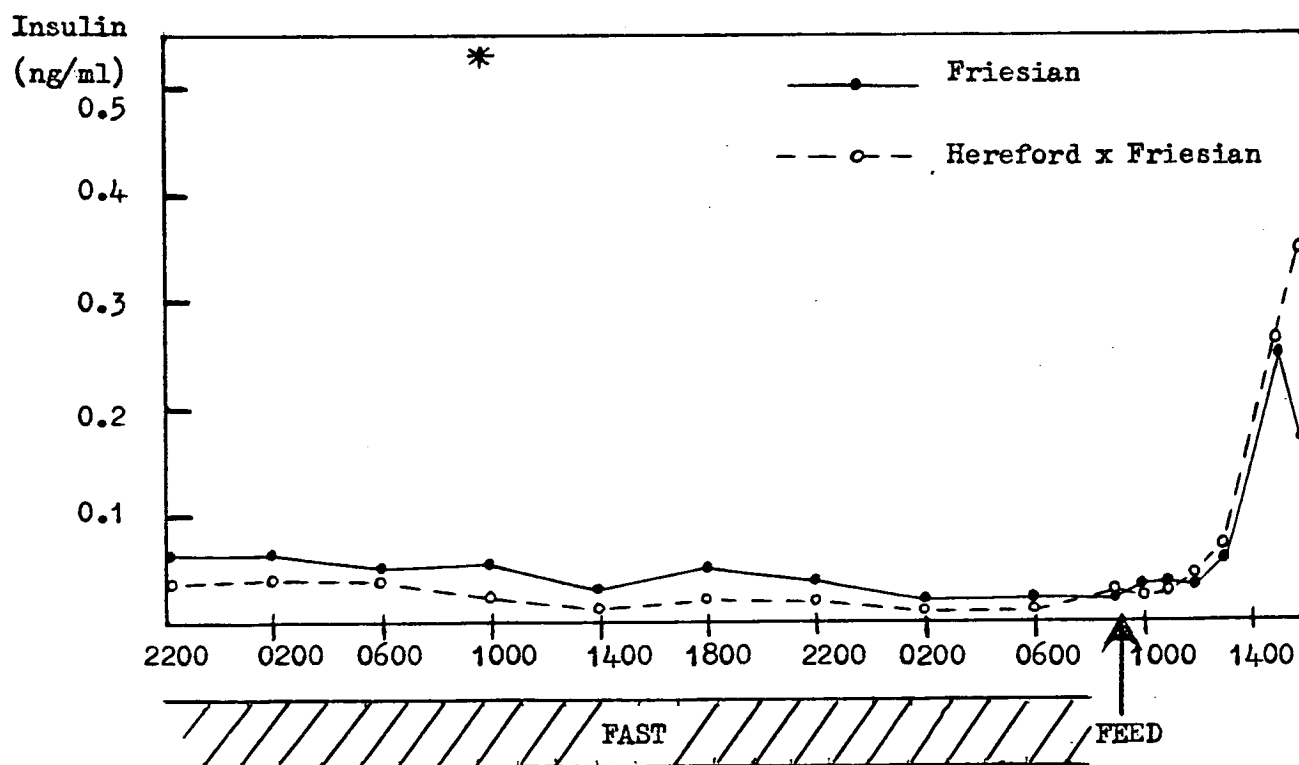


Table 5.6 :

Mean hormonal responses to injections of sodium propionate in dairy and crossbred calves aged 16 weeks (postweaning).

Insulin (ng/ml)

Breed :	Friesian		Hereford x Fr	
Dose :	High	Low	High	Low
Time 1	1.236	0.655	3.340	2.488
Time 2	0.892	0.209	1.056	0.291
Time 3	0.413	0.025	0.290	0.082

Growth Hormone (ng/ml)

Breed :	Friesian		Hereford x Fr	
Dose :	High	Low	High	Low
Time 1	-18.3	-10.9	-15.3	-21.1
Time 2	- 0.8	- 9.6	+ 1.7	-18.9
Time 3	- 5.5	+32.4	+ 2.1	+12.0

High dose = 1 mmol/kg body wt.

Low dose = 0.5 mmol/kg body wt.

Time 1 = 3 hours after withdrawal of food.

Time 2 = 24 hours after withdrawal of food.

Time 3 = 48 hours after withdrawal of food.

3 animals per treatment per breed; dose sequence randomized.

Table 5.7 :

Analysis of variance for insulin responses to propionate injection in dairy and crossbred calves aged 16 weeks (postweaning).

Source	d.f.	MS	F
Breed	1	4.616	2.94 , $P < 0.20$
Animals within breed	10	1.565	1.50
Times	2	9.926	9.55 , $P < 0.01$
Doses	1	4.926	4.74 , $P < 0.05$
Breed x Times	2	3.344	3.21 , $P < 0.10$
Residual	18	1.039	

Table 5.8 :

Analysis of variance for growth hormone responses to propionate injection in dairy and crossbred calves aged 16 weeks (postweaning).

Source	d.f.	MS	F
Breed	1	206.2	0.66
Animals within breed	10	310.2	2.27
Times	2	2419.9	15.55 , $P < 0.001$
Breed x Times	2	24.1	0.17
Breed x Doses	1	893.9	6.56 , $P < 0.05$
Times x Doses	2	1039.0	7.62 , $P < 0.01$
Residual	15	136.2	

Table 5.9 :

Within-breed correlations among mean hormone levels, body weight and weight gain in male calves at 6 weeks of age (preweaning).

	T4	T3	Insulin	GH	Body wt.	Gain 2-6	Gain 2-16
T4	1.000						
T3	0.859***	1.000					
Insulin	-0.042	-0.290	1.000				
GH	-0.157	-0.076	0.319	1.000			
Body weight	0.038	0.274	-0.211	0.228	1.000		
Gain 2-6 weeks	-0.028	0.095	-0.259	0.106	0.857**	1.000	
Gain 2-16 weeks	-0.225	-0.165	-0.061	0.103	0.848**	0.854**	1.000

Table 5.10 :

Within-breed correlations among mean hormone levels, body weight and weight gain in male calves at 16 weeks of age (postweaning).

	T4	T3	Insulin	GH	Body wt.	Gain
T4	1.000					
T3	0.887**	1.000				
Insulin	0.210	0.175	1.000			
GH	-0.071	-0.205	-0.841**	1.000		
Body weight	0.711**	0.816**	0.210	-0.286	1.000	
Gain 2-16 weeks	0.634*	0.714**	0.243	-0.35-	0.969**	1.000

Table 5.11 :

Within-breed correlations among mean hormone levels, body weight and weight gain measured in male calves at 6 weeks of age and the same traits measured at 16 weeks of age.

	T4 (16 weeks)	T3 (16 weeks)	INS (16 weeks)	GH (16 weeks)	Body wt. (16 weeks)	Gain (2-16 weeks)
T4 (6 weeks)	0.271	-0.015	-0.461	0.830**	-0.162	-0.225
T3 (6 weeks)	0.239	0.054	-0.576*	0.794**	-0.036	-0.165
Insulin (6 weeks)	0.207	0.127	0.742**	-0.455	-0.063	-0.062
GH (6 weeks)	0.252	0.105	0.722**	-0.477	0.165	0.103
Body wt. (6 weeks)	0.718**	0.725**	0.035	-0.082	0.916**	0.848**
Gain (2-6 weeks)	0.531	0.441	0.043	-0.145	0.794**	0.854**

* = $P < 0.05$, ** = $P < 0.01$

D.f. = 9

Correlation between insulin response to propionate at 6 versus 16 weeks
= $r = 0.554$ on 9 d.f.

V.4 Discussion.

V.4.1 The thyroid hormones.

Levels of T₄ and T₃ in parts I and II were very similar to those reported by Kahl et al. (1977) at the corresponding ages, although distributed about a lower mean than those of adult bulls (see chapter III) or euthyroid humans. Moderately high repeatabilities were obtained for both hormones at both ages (0.45-0.68), again similar to previous reports (Bard, 1973; Joakimsen, 1975). Mean levels of the two hormones over 24 hours were strongly correlated at both ages to the extent that measuring both hormones may be superfluous provided that the mean level of one or the other can be determined sufficiently accurately. This is probably because much T₃ is formed in peripheral tissues by monodeiodination of T₄ rather than being secreted by the thyroid gland. Within animals, however, T₄ and T₃ levels varied asynchronously over the 24 hour period, perhaps implying a time-lag between the two hormones.

The consistent but non-significant breed difference in both hormones at 6 weeks of age was not evident at 16 weeks; this may have been due to the very low levels of animal no. F3, a weak Friesian calf which died between parts I and II. Webster, Gordon and MacGregor (1978) measured heat production in Friesian and Hereford x Friesian calves from birth to eight weeks old and found no significant breed difference such as might have been expected if T₄ levels were different and exerting different calorific effects.

During fasting, levels of T₄ fell slightly and levels of T₃ considerably. This has been previously observed in man (Carlson, Drenick, Chopra and

Hershman, 1977; Spaulding, Chopra, Sherwin and Lyall, 1976). This appeared to be due to reduced carbohydrate intake leading to less formation of T3 in peripheral tissue. On refeeding, T3 levels increased, again in accordance with previous reports (Spaulding et al., 1976). It is possible that the reduction of T3 levels conserves energy by lowering metabolic rate.

Bermudez, Surks and Oppenheimer (1975) found a high incidence of low T3 levels in patients with non-thyroidal disease. The present study provided some evidence to confirm this, with the weakest and lightest calves at both ages having the lowest levels of T3 and also T4.

There were strong positive correlations of both T3 and T4 levels with body weight and with weight gain at age 16 weeks, though not at 6 weeks. The absence of these correlations in part I may have been due to erratic weight changes during the settling-in period or to the feeding regime (restricted rather than ad libitum) which may have masked differences. These correlations were only with the liveweight gain prior to the age of measurement; no correlation was observed between levels of T4 or T3 at 6 weeks and liveweight gain up to 16 weeks. It appears, therefore, that the thyroid hormones are not controlling future growth; instead it seems that they act to maintain some form of caloric homeostasis, being reduced during fasting and in disease to conserve energy, and being increased when weight gain and presumably food intake have been high to increase metabolic rate. Bard (1973) found a significant correlation between daily gain on test and T4 levels at the end of test. There is thus no evidence that T4 or T3 levels are useful predictors of growth

rate; in the present study the best predictor of weight gain in the second period was the gain during the first period.

Although Joakimsen (1975) reported a small positive correlation between T3 levels of young bulls and their daughters' milk yield, no significant breed difference was observed in the present study despite the large expected difference in genetic merit for milk production between crossbred and dairy animals. The tendency was for the Friesians to have lower levels, particularly in part I. There was therefore no support for the idea that thyroid hormone levels might be used as criteria for indirect selection of superior dairy bulls.

V.4.2 Growth hormone.

Large variation occurred in GH levels, as reported by Bassett (1974), and low repeatabilities were obtained. Nevertheless it was possible to detect significant differences among animals within breed; Hove and Blom (1973) found a similar effect.

Levels of GH fell significantly during fasting and rose significantly on refeeding at both ages. This was slightly unexpected since GH levels have been found to be elevated in cows in a state of energy deficit, e.g. during early lactation (Hart et al., 1975; Hove, 1974). McAtee and Trenkle (1971) found a slight reduction in GH levels in four 5-month old crossbred heifers during a 48 hour fast. In the present study, the smallest depression of GH levels occurred in the crossbred animals at 16 weeks, so that these results are perhaps comparable. Nevertheless the physiological significance of the fall during fasting and rise after refeeding is not clear.

The responses of GH to the injections of energy metabolites were variable at both ages. At age 6 weeks neither glucose nor propionate produced a significant response, although glucose is known to depress GH levels in man (Glick, Roth, Yalow and Berson, 1965). At age 16 weeks the responses were inconsistent, with the propionate injections tending to depress GH levels in fed animals but to raise GH levels when given after 48 hours' fast. McAtee and Trenkle (1971) found a slight but non-significant depression in GH levels after propionate injection following 24 hours' fast (corresponding to time 2 in the present study).

GH is known to have an anabolic effect whereby it promotes protein deposition (Tanner, 1972). Injection of exogenous GH was shown to increase daily gain and leanness in pigs on both ad libitum and restricted feeding regimes (Machlin, 1972). In the present study the correlation between mean GH levels and body weight was negative, particularly in part II when the correlation was -0.741 if animal no. 7 was excluded. On the other hand, GH levels at 6 weeks showed a small positive correlation with liveweight gain thereafter.

The physiological significance of the various GH responses to propionate is unclear. The size of the (negative) responses decreased the longer the period of fasting prior to the propionate injection, just as the size of the (positive) insulin responses decreased. This indicates that fasting, by altering energy status, affects not only basal levels of GH as observed in the previous week (see figure 5.10) but also its patterns of secretion. The breed x dose and the time x dose interactions suggest, if true, that GH secretion is controlled by an interplay of factors. It has been shown

that injection of glucose can reduce a rise in GH levels caused by depression of free fatty acids (Quabbe, Ramek and Luyckx, 1977). It appears, therefore, that the response depends both on the levels of circulating energy metabolites, which change with fasting, and on the quantity injected. During fasting the Friesians showed a greater drop in GH levels than the crossbreds during the first 24 hours : this may relate to the breed x dose interaction. Overall, however, since neither breed nor dose effects were significant, the results are difficult to interpret.

Tucker et al. (1974) measured GH in dairy cows and bulls and found low repeatability and no correlation with breeding value for milk production. They concluded that simple basal estimates of GH were insufficient for use in selection processes unless sampling variation was more completely defined or controlled, and that "application of suitable stimuli" might offer a more fruitful approach. In the present study, the repeatability was also low; and although sufficient samples were taken to establish a significant between-animal effect, the breed difference was non-significant. The application of stimuli in the form of propionate injections did produce responses, but these were inconsistent and were, if anything, negatively correlated with basal levels (the mean levels over 24 hours). These results indicate that despite the difference in GH levels between the same two breeds in early lactation (Hart et al., 1975), this difference is not readily detectable in male calves of these ages. They therefore tend to reaffirm the conclusions of Tucker et al. (1974) as stated above concerning the possible usefulness of GH as a selection criterion for milk production.

V.4.3 Insulin.

In part I of the experiment when the calves were meal-fed three times a day on milk substitute, it can be seen from figure 5.4 that the main diurnal variations were prandial as has been reported previously for cattle (Hove and Blom, 1973) and sheep (Bassett, 1974). Also, the crossbred calves showed a larger rise in insulin after meals as indicated visually in figure 5.7 and as demonstrated by the significance of the breed x time interaction (see table 5.1). This breed difference closely parallels the results of Hart et al. (1975) who similarly found a rise in the insulin levels of lactating crossbred heifers after feeding but no rise in those of dairy heifers.

In part II, the elevated insulin levels of both breeds between samples 10 and 17 (1700 hrs to 2400 hrs) cannot definitely be ascribed to prandial variation because of the ad libitum feeding regime. However, behavioural observations on cows fed a complete diet ad libitum have shown that 75% of rumination is performed outwith the hours of daylight, unlike meal-fed cows which indulge in rumination after each meal (Lees, 1976; Gordon and McAllister, 1970). It may be, therefore, that the absorption of nutrients from the gut follow the pattern of rumination more closely than the pattern of eating. This hypothesis, that there may be a time lag between eating and absorption of nutrients, was supported by the results from fasting and refeeding (see figure 5.11). Insulin levels dropped after 12 hours fasting and remained low as previously reported (McAtee and Trenkle, 1971); food was replaced after 51 hours fast at 0900 hrs but insulin levels did not rise until at least 5 hours later.

In addition to the responses to meals in part I, insulin levels also rose in response to injection of propionate and these responses were significantly correlated with each other (see table 5.3). The lack of response to glucose injection was somewhat surprising since calves pre-weaning have monogastric digestion similar to non-ruminants where glucose normally elicits an insulin response. It is possible that the response to glucose was too brief to be detectable in the sample 20 minutes after injection. In part II, although the calves were not meal-fed, several treatments were used for the propionate injections and the results obtained were as follows (and see tables 5.6 and 5.7) : the dose of propionate injected had a significant effect on the insulin response, the high dose giving a larger response. Length of fasting (Time) also had a significant effect with the size of the response being much greater in the fed than in the fasted animals. Hove (1978) found a similarly reduced insulin response to glucose infusion after 48 hours fasting when compared with fed animals. The crossbred calves showed consistently larger responses than the dairy calves for all dose-time combinations and particularly in the fed animals. It is suggested that this breed difference is comparable with the different insulin responses to feeding in the two breeds as observed in part I and by Hart et al. (1975; see above). These patterns of insulin secretion in response to food may directly determine the fate of absorbed energy metabolites, with high levels of insulin leading to energy storage as body tissue. This hypothesis is consistent with the observations of Hart et al. and the following evidence : that insulin levels fall after the start of lactation (Hove, 1974); that insulin levels are negatively correlated with milk yield (Tucker, 1973); and that injection of insulin during lactation depresses milk yield (Kronfeld et al., 1963).

If the breed difference in insulin response to incoming nutrients does underlie their different capacities for milk production, it is possible that some test of this sort could be developed as a selection criterion; but considerable caution must be used when interpreting the results of a comparison between breeds in terms of the situation within a breed. Nevertheless, further investigation of this subject seems warranted (see chapter VI), particularly since the correlations obtained among insulin responses to meals and to propionate injection in part I (see table 5.3) suggest that their repeatability may be moderately high. Also, the correlation between the response to propionate at 6 weeks and that at 16 weeks ($r = 0.554$) suggest that such a test might be applicable either preweaning or postweaning.

Gregory et al. (1976) reported strong correlations in pigs between fasting insulin levels and fatness. In the present study no carcass characteristics were estimated but only small correlations between insulin and weight gain were observed.

V.4.4 Conclusions.

Most of the results have been discussed except for three of the correlations in table 5.11. Strong positive correlations were found between mean T4 levels in part I and GH levels in part II; similarly for T3 in part I versus GH in part II; and for GH in part I versus insulin in part II. None of these has obvious physiological significance and there are no obvious reasons for bias. If they are not chance effects, it must imply that high levels of, say, T4 lead to some physiological state which

directly or indirectly causes GH levels to be elevated 10 weeks later. Since no measurements except body weight were taken between parts I and II there is no information on intermediate physiological status and the reasons for these three correlations cannot be easily deduced.

In terms of physiological aids to selection, probably the most interesting result is the breed difference in insulin response to a nutritional stimulus, i.e. a meal or a propionate injection, as described above. The use of nutritional stimuli was particularly vindicated : first, because the breed difference in mean insulin levels was not easily detectable over the 24 hour period; and second, because the various insulin responses were correlated with each other and with the mean levels over the 24 hours. It appears that detailed investigation of the dynamics of hormone secretion, rather than of the basal state, can in some cases reveal differences which may be relevant to production traits by virtue of their effect on energy partition.

CHAPTER VI GENERAL DISCUSSION AND CONCLUSIONS.

VI.1 Discussion of conclusions from chapters III, IV and V.

In experiment 1 (chapter III), insulin, T₄ and T₃ were measured in two samples from each of 178 Friesian bulls undergoing progeny tests for milk production. The relationship between hormone levels and progeny test results was examined but the regressions of hormone levels on ICC were not significant for any of the three hormones and the correlations with ICC were near zero. In addition, environmental effects were significant for each hormone : the effects of station for insulin and T₄, of age and time after feeding for T₃, and the effect of time of day on T₄ and insulin.

It could be argued that since the repeatability estimates were low for all three hormones the regressions on ICC might have become significant if more than two samples per bull had been taken and the error variance thus reduced; but it can be shown (see note at the end of this section) that even with a large number of measurements per bull the correlations between hormone levels and ICC would be expected to lie in the range of 0.0 ± 0.20 in each case.

Although Joakimsen et al. (1971) found a significant positive correlation between thyroxine degradation rate and breeding value in dairy bulls, there was no indication of such a relationship between either T₄ or T₃ and ICC in the present study. Joakimsen considered that plasma levels of T₄ alone did not really reflect the biological role of the thyroid hormones. The present study would support his conclusion and would suggest that estimates of basal plasma levels of T₄, T₃ or insulin in isolated samples are not genetically related to breeding value.

In experiment 2 (chapter IV), insulin, T3 and T4 were measured in Friesian and Jersey calves which had either very high or very low expected merit for milk production. No significant differences between the 'high' and 'low' groups of calves was found either within or across breeds. As in experiment 1 the repeatability estimates were low (0.04 - 0.17) and the effects of age on T3 and insulin and of season on T4 and insulin were significant. This experiment tends to support the conclusion from experiment 1 that basal levels of these hormones do not appear to be related to genetic merit for milk production.

In experiment 3 (chapter V) six Friesian and six Hereford x Friesian calves were studied, both before and after weaning, using an intensive sampling régime and a variety of nutritional treatments. Sufficient samples were taken from each animal to be able to identify significant between-animal effects for all the hormones studied at both ages. Nevertheless none of the breed differences in 'basal' hormone levels over 24 hours quite reached significance at the 5% level, despite the large expected difference between the breeds in merit for milk production. The main differences observed between the two breeds was in their patterns of insulin secretion. The crossbred calves showed a considerably greater rise in insulin level after meals at age six weeks than did the dairy calves : they also showed a larger insulin response to injection of sodium propionate both at ages 6 and 16 weeks. The magnitude of the insulin responses to meals or to injection of propionate was correlated with mean basal insulin levels : also, mean insulin levels at age 6 weeks were correlated with mean levels at 16 weeks ($r = 0.74$).

The main conclusion from this experiment, therefore, was that the use of an intensive sampling régime, while not necessarily contributing greater

accuracy than, say, five samples per animal, does have the advantage that it is possible to study the dynamics of hormone secretion as well as the basal condition. This led to the observation in experiment 3 of the apparent breed difference in response of insulin to an energy stimulus.

The repeatability estimates for T3 and T4 in experiment 3 were higher (0.45 to 0.68) than those for experiments 1 and 2. This is probably because the hourly measurements have common environmental variance, especially since the biological half-lives of both hormones are thought to be longer than 1 hour and perhaps as much as 24 hours (Sterling and Lazarus, 1977). Perhaps more suitable figures for comparison are the correlations between mean T3 levels and T4 levels at 6 weeks versus those at 16 weeks which were 0.054 and 0.271 respectively. The half-lives of insulin and growth hormone are both very short and the repeatability estimates were low (0.07 to 0.20); but mean insulin levels at the two ages were highly correlated (0.74). This suggests that it is possible to characterise animals' insulin levels accurately provided sufficient samples are taken.

Note on section VI.1 :

If t is the correlation between measurements of the same individual (repeatability), the variance of the mean of n measurements can be expressed as a proportion of the variance of one measurement as follows :

$$\frac{V_{p(n)}}{V_p} = \frac{1 + t(n-1)}{n} = t + \frac{1}{n} (1-t) \quad \text{Falconer (1960)}$$

If the correlation between x and a variate y , measured once, is

$$r_{x,y_i} = \frac{\text{Cov}(x,y)}{\sqrt{V(x) \cdot V(y)}}$$

Then the correlation between x and y measured n times is

$$r_{x,\bar{y}_n} = \frac{\text{Cov}(x,y)}{\sqrt{V(x) \cdot V(y) \cdot \left[t + \frac{1}{n} (1-t) \right]}}$$

and as n becomes large the correlation becomes

$$r_{x,\bar{y}^\infty} = \frac{\text{Cov}(x,y)}{\sqrt{V(x) \cdot V(y) \cdot t}} = \sqrt{\frac{1}{t}} \cdot r_{x,y_i}$$

$$\text{Also, } r_{x,\bar{y}^\infty} = \sqrt{\frac{(1-t) + \frac{1}{n}}{\frac{1}{nt}}} \cdot r_{x,\bar{y}_n}$$

In experiment 1, the lowest repeatability estimate was that for insulin

($t = 0.10$). Thus r_{x,\bar{y}^∞} for insulin is $5.5 \times r_{x,\bar{y}_2} = 0.19$.

Similar calculations of r_{x,\bar{y}^∞} for T4 and T3 give values of -0.16 and -0.087 respectively.

VI.2 Application of a physiological selection criterion for milk production.

Hinks (1971) has shown that the adoption of indirect selection for milk production is unlikely to be justified at the sire level unless both the heritability (h^2) of the secondary character and its genetic correlation (r_G) with breeding value were both very high. If the secondary character were measureable in males less than one year old, the sire-son generation interval could be reduced from about 8 years under the present U.K. scheme to about 2 years; but despite this possible gain it would still seem unlikely that the replacement of the progeny test by indirect selection could be envisaged.

Falconer (1960) writes : "The most effective use that can be made of a correlated character is in combination with the desired character, as an additional source of information about the breeding value of individuals". Under the present U.K. system, it is possible to envisage a physiological character being used in this way as a 'preselection' criterion with which to screen young bulls as they enter the testing programme before the bulk of the costs are incurred. The adoption of such a criterion would depend on several factors, among them 1) ease of measurement in terms of costs and procedures : 2) whether measureable at a young age : 3) whether the h^2 and r_G were sufficiently high to provide extra discrimination among half-brothers over and above that provided by bull dam selection on pedigree and phenotype. Because the costs involved in progeny testing are considerable, the possibilities of screening more young bulls at small extra expense, or of reducing the number of bulls on test while maintaining the selection differential, both represent opportunities for increasing rate of genetic gain per unit cost.

In the context of the present study, the results of experiments 1 and 2 show that simple estimates of basal hormone levels, at least of thyroxine, triiodothyronine and insulin do not have sufficiently high h^2 or r_G to be considered as physiological selection criteria in themselves. The results of experiment 3 suggest that measurements of hormones after imposition of suitable treatments may be more closely related to milk production, as concluded by Tucker et al. (1974) who studied growth hormone and prolactin. Possible methods of investigating this idea further are discussed in the next section.

VI.3 Prospects for further research.

The current project has recently been extended by a further experiment. It was decided to study the within-breed difference between two groups of contemporary calves, one group having high expected merit for milk production and the other group low expected merit. In all, 40 Friesian calves (20 high, 20 low) were studied under a range of nutritional conditions using procedures similar to those described for experiment 3 part II. In addition to T₄, T₃, insulin and growth hormone, a number of energy metabolites was measured : urea, free fatty acids (FFA), glucose, β -hydroxybutyrate, albumin and total protein (Tilakaratne, Alliston, Carr, Land and Osmond, 1979). Differences in metabolite levels were found between the two groups of calves, particularly during fasting : the progeny of the high ICC bulls had significantly lower levels of urea and higher levels of FFA than the low ICC group. There was also a tendency for the high ICC group to have a higher level of T₄ which approached significance at the 5% level. No difference was observed between the two groups in insulin responses to feeding to correspond with the breed difference observed in experiment 3. There are several reasons which may account for this; first, this experiment was carried out after weaning whereas the breed difference in experiment 3 was only observed before weaning; second, the animals had access to hay ad libitum which may have affected their eating patterns; and third, the within-breed difference in milk production may have a different physiological basis from the between-breed difference. Nevertheless, the differences between the high and low groups in metabolite levels and the fact that these were most significant during fasting tended to support the contention that the changes in levels of hormones or metabolites may be more important, in terms of genetic differences, than their basal concentrations; and that the use of stimuli such as nutritional treatments may induce the expression of this variation.

Further studies are being carried out to determine whether differences in urea and FFA after fasting and in insulin after feeding are repeatable in an on-farm environment at different ages.

As regards future prospects, further basic physiological research is needed before genetic differences can be understood and placed in context. For instance, although it still seems likely that differences in milk yield in cattle arise from differences in energy partition and that these are to some extent genetically determined, the physiological mechanisms controlling the fate of ingested nutrients are not well understood; and although some studies have shown relationships between the breeding value of dairy bulls for milk production and physiological parameters, e.g. thyroxine degradation rate (Joakimsen et al., 1971) and urea (Stark et al., 1978), the reasons for these relationships are still not clear.

Perhaps the most immediate need is for further research into the complex way in which hormones and metabolites interact to control the rates of protein synthesis and breakdown and of lipid synthesis and breakdown. This can best be done by applying recent techniques for quantitative measurement to at least one of these processes. Although such work has been started using rats, it is likely that results for ruminants would be quite different. Such studies should aim to clarify the manner in which genetic, nutritional and other environmental factors affect not only milk yield but growth, feed efficiency, fatness, leanness, milk quality and other traits.

In genetic terms, it seems likely that the physiological differences between animals tend to be expressed more in 'dynamic' situations, that is in response to changes in conditions or to imposed treatments. The results

from the present project seem to suggest that survey-type studies suffer from two disadvantages : first, that genetic differences among 'basal' levels tend to be smaller and, second, that environmental effects can be large, particularly with few measurements per animal. If this is so, the most fruitful approach will involve multiple sampling, with as great a degree of environmental control as possible, in order to identify changing patterns of hormone and metabolite levels. For these reasons ABRO has set up 'high' and 'low' lines of dairy cattle in which it is hoped physiological differences may continue to be studied. Some experiments which could be considered are

- 1) whether high and low animals do differ in insulin response to feeding and at what ages
- 2) whether high and low animals do differ in responses of urea and FFA to fasting and at what ages
- 3) whether the frequency of meals affects patterns of insulin secretion and hence growth rate, fatness or milk yield
- 4) an assessment of the role of glucagon, for which assay techniques have only recently become available.

In the more distant future perhaps most is to be gained by quantitative measurement of protein synthesis and breakdown as described above.

As mentioned above (section VI.2), the circumstances in which a physiological selection criterion would be most applicable is probably in the screening of young bulls prior to progeny testing. In the U.K., the group of animals corresponding to these are the bull calves at the MMB Bull Rearing Unit, Chippenham, Wiltshire, where the calves are collected and held from about 3 to 15 months of age before entering the progeny testing programme. It may be hoped that an experimental procedure for investigating a possible physiological selection criterion can be developed as soon as possible for testing on these animals.

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